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The cardiovascular protective effects of rooibos (*Aspalathus linearis*) extract on diesel exhaust particles induced inflammation and oxidative stress involve NF-κB- and Nrf2-dependent pathways modulation

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Abstract

Studies have shown that diesel exhaust particles (DEP) induced oxidative stress and inflammation. This present study examined the molecular effects of aqueous rooibos extract (RE) on the cardiovascular toxic effect of methanol extract of DEP in exposed Wistar rats. The results showed that DEP caused significant ($p < 0.001$) increase in MDA and CDs levels in the aorta and heart but this increase was significantly ($p < 0.001$) attenuated by rooibos extract. DEP

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induced IL-8, TNFα, IL-1β and decreased IL-10 gene expressions, all of which were reversed in the presence of rooibos extract. The expression of NF-κB, and IκKB genes were also significantly ($p < 0.001$) induced by DEP in both tissues, but pre-treatment with RE attenuated these effects. In contrast, DEP repressed IκB mRNA level, which was significantly ($p < 0.001$) reversed by rooibos extract pre-treatment. In addition, pre-treatment with rooibos extract attenuated the increased Nrf2 and HO-1 mRNA levels caused by DEP. This indicates the potential of rooibos extract to protect against DEP-induced cardiovascular toxicity.

Keywords: Cell biology, Molecular biology, Pharmaceutical science

1. Introduction

Exposure to air pollution has been reported in many epidemiological and experimental studies to be responsible for the increase incidence in cardiovascular diseases and atherosclerosis (Brook et al., 2010; Møller et al., 2011; Araujo and Nel, 2009). In addition, increased incidence of Ischemia strokes, acute myocardial infarction, and congestive heart failure have been linked to acute exposure to air particulate matter (Lawal et al., 2016; Araujo and Nel, 2009).

Diesel exhaust particles (DEP) is an important component of air particulate pollutants, released into the atmosphere from diesel exhaust emission of diesel engines from industrial, vehicular, and anthropogenic activities as well as from residential homes (Araujo and Nel, 2009; Lawal and Araujo, 2012). Both in vitro and in vivo studies have shown that oxidative stress and inflammation are major mechanisms in DEP toxicity (Lawal et al., 2015, 2017, Montiel-Davalos et al., 2010; Tobwala et al., 2013; Robertson et al., 2012; Miller et al., 2013). DEP is heterogeneous in composition consisting of polycyclic aromatic hydrocarbons (PAHs), elemental carbon, heavy metals, and soots (Lawal et al., 2016). The polycyclic aromatic hydrocarbons (PAHs) have been implicated as the major constituents responsible for most of the adverse effects of DEP (Totlandsdal et al., 2012, 2014).

Although the major route of exposure to DEP is via intranasal into the lungs, substantial amounts are deposited on the skin surface, which may result in the leaching of their chemical constituents into the keratinocytes (skin cell). DEP, as an environmental pollutant, can interact directly with the epidermal skin layer, which forms the interface between the internal and external environment Dang et al. (2008). Indeed, studies have shown that DEP deposited on the skin of a healthy subject gets internalized by both monocyte-derived macrophages from peripheral blood and keratinocytes to promotes pro-inflammatory, pro-oxidative and pro-fibrotic responses in normal human skin (Fiorito et al., 2011; Dang et al., 2008).

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Several studies have shown that the use of antioxidants can protect against the DEP-induced toxicity (Tseng et al., 2015; Frikke-Schmidt et al., 2011; Yin et al., 2013). We have shown that both genetic and pharmacological modulation of heme oxygenase-1 (HO-1) protects against DEP-induced oxidative stress and inflammation in human microvascular endothelial cells (Lawal et al., 2015). However, the uses of antioxidant phytochemicals against DEP toxicity have not been well-established.

One important source of such antioxidants is Rooibos (*Aspalathus linearis*), a native plant of South Africa accounting for more than 80% of the total plant species in the Cape Floral Kingdom (Joubert et al., 2008) and its readily available in commercial pack in many homes around the world. Rooibos has gained attention recently as a medicinal plant due to its antioxidant, cancer modulating, antimutagenic and cardiovascular health properties (Pantsi et al., 2011; Marnewick et al., 2005, 2009). Indeed, its health promoting ability has been well-established in experimental animals (Marnewick et al., 2009, 2005).

This present study examined the protective effect of aqueous extract preparation of commercially available rooibos tea on DEP-induced oxidative stress and inflammation in cardiovascular tissue of wistar rats, in order to proffer therapeutic solution to ameliorating the toxic effects of DEP and thus prolong life span.

2. Materials and methods

2.1. Chemicals

Cyclohexane, 5-5'''-thio-2-nitrobenzoic acid (DNTB), dithiothreitol (DTT), DMSO, Thiobarbituric acid (TBA), butylated hydroxyltoluene (BHT), nicotinamide adenine dinucleotide phosphate reduced (NADPH), reduced glutathione (GSH), chloroform, methanol, ortho-phosphoric acid, coomassie brilliant blue were obtained from Sigma-Aldrich (Germany). Nuclease free water was obtained from VWR life science (Solon, USA). Primers were obtained from Inqaba biotechnology (Hatfield, South Africa). TRI Reagent® was purchased from Zymo Research (USA). ProtoScript II First Strand cDNA Synthesis Kit and Luna Universal qPCR Master Mix were bought from BioLabs (New England).

2.2. Rooibos extract preparation

A pack of commercially available Freshpak® Rooibos Tea (Batch # 6001156905236, Entryce Beverages, South Africa) was brought from Shoprite shopping mall, Akure, Ondo State, Nigeria. The pack contains 20 tagless tea bags, were cut open with a sterile scissor, and the contents weighed and boiled in water (1 g/20 ml boiling water) for 15 min. The boiled content was allowed to

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cool at room temperature and then filtered through Whatmann paper into a new sterile beaker. The filtrate was lyophilized and the sample was stored at -20 °C for further use. Before use, the lyophilized sample was reconstituted at 10 mg/ml stock solutions in 0.9% saline and kept at -20 °C.

2.3. Diesel exhaust particles (DEP) collection and methanol extract preparation

DEP was collected from the exhaust of a 1996 model, 146 hpsix-cylinder MAN tipper truck engine (engine # G0967) at a tipper garage in Akure, Ondo State, Nigeria as described by Li et al. (2004). The particles were collected on a high capacity glass-fiber filter and scraped into a glass container for storage. The particles powder was stored in the glass container at -80 °C away from light for further use. The methanol extract of the DEP was prepared as previously described (Lawal et al., 2015). Briefly, DEP was suspended in methanol (100 mg/25 ml methanol) and sonicated for 5 min on ice. The suspension was then centrifuged at 4 °C for 10 min at 2500 x g. The methanol supernatant was transferred into a new polypropylene tube and re-centrifuged for 10 min at 2500 x g at 4 °C. The supernatant was collected in a pre-weighed tube and dried under nitrogen gas. The dried DEP extract was then reconstituted in DMSO (100 μg/μl DMSO) and stored at -80 °C away from light.

2.4. GC-MS analysis of the polycyclic aromatic hydrocarbons (PAHs) contents in DEP extract

GC-MS was carried out using HP6890 series gas chromatography with a HP-5 capillary column coated with 5% phenylmethylsiloxane stationary phase for compounds separation as previously described (El-Bazaoul et al., 2011). The chromatography was coupled to an inert mass spectrometer and samples were injected at a volume of 1 μl, total flow rate of 25.8 ml/min and temperature of 260 °C. The retention time was marched with the reference standard to identify the compounds of interest. All analyses were done in triplicate.

2.5. In vitro determination of the redox (oxidative) potential of methanol extract of DEP

The redox (oxidative) potential of the DEP extract was determined by the method described by Charrier and Anastasio (2012). The method was based on indirect monitoring of the rate of dithiothreitol (DTT) consumption by redox-active chemicals. The redox-active chemicals oxidised DTT to the disulphide form, and this was monitored by measuring the rate of 5-thio-2-nitrobenzoic acid (TNB) formed at 412 nm, in a reaction between DTT and DNTB (5,5′-thio-2-nitrobenzoic acid). Briefly,

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50 μl of methanol extract of DEP (or DMSO) was added to 3.0 ml of 100 μM DTT (prepared in 0.10 M phosphate buffer, pH 7.4) in a testtube. The mixture was shaken vigorously in water bath at 37 °C. 500 μl of this solution was taken into a new testtube at different time (0, 5, 10, 15, 20 and 30 min) interval. 500 μl of 10% trichloroacetic acid (TCA) was added to stop the reaction at each time interval. 50 μl of 10 mM DTNB (prepared in 0.10 M phosphate buffer, pH 7.4) was added, mixed thoroughly and allowed to react for 5 min. 2.0 ml of 0.40 M Tris-Base (pH 8.9) was added to the mixture and TNB formation was monitored by measuring absorbance at 412 nm against the sample blank containing DMSO alone. TNB levels were quantified using the extinction coefficient of 14150 M^{-1} cm^{-1}. The redox-activity of the extract was expressed as the rate of DTT loss in μM/min.

2.6. Animal treatment and tissues collection

All animal studies were performed according to approved protocols of the Animal Ethics Committee of School of Sciences, Federal University of Technology Akure (FUTA). Male wistar rats (6 weeks old) weighing 80−100 g were purchased from local animal vendor in Akure, Ondo State, Nigeria. The rats were housed in the biochemistry departmental animal house in FUTA. The animals were given free access to food (growers poultry feed, Vital Feed Nigeria Limited) and water. The rats were divided into 4 experimental groups of 6 rats/group: control group was given saline orally for 4 weeks and DMSO subcutaneously twice a week in the final 2 weeks of treatment; group 2 was given 50 mg/kg aqueous rooibos extract (RE) orally for 4 weeks and DMSO subcutaneously twice a week in the final two weeks; group 3 was given 50 mg/kg RE for 4 weeks orally and 0.7 mg/kg DEP extract subcutaneously for twice a week in the final two weeks; and Group 4 was given oral saline for 4 weeks and 0.7 mg/kg DEP extract subcutaneously for twice a week in the final two weeks. The rats were sacrificed 24 hr after the last treatment and blood were withdrawn from the heart. The aorta and heart biopsies were collected and stored at -80 °C for subsequent analysis. Protein content of the tissue homogenate was determined by Bradford method (1976).

2.7. Lipid peroxidation assay

Thiobarbituric acid reactive substances (TBARS) were used to quantified lipid peroxidation as previously described (Yagi, 1976). Briefly, 6.25 μl of 4 mM cold butylated hydroxyl toluene (BHT) (prepared in ethanol) and 50 μl of 0.2 M orthophosphoric acid were added to 50 μl sample and vortexed briefly. 6.25 μl of 0.11M thiobarbituric acid (TBA) (freshly prepared in 0.1 M NaOH) was added to the mixture and briefly vortexed. The mixture was then incubated at 90 °C for 45 min and latter cooled on ice for 2 min. The mixture was further left at room temperature for 5 min before the addition of 500 μl n-butanol and 50 μl saturated NaCl. The

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mixture was briefly mixed and then centrifuged at 12,000 x g for 2 min at 4 °C. About 400 μl of the upper butanol phase was transferred into 1 ml cuvette and absorbance was taken at 532 and 572 nm background using GS-UV 32 PCS spectrophotometer (General Scientific). The MDA level was calculated using the extinction coefficient of 154000 M^{-1} cm^{-1}. The results were expressed as mmole MDA/mg protein.

2.8. Conjugated dienes levels determination

The levels of conjugated dienes (CDs) in the tissue homogenates were determined as previously described (Buege and Aust, 1978). Briefly, 500 μl samples were added to 1.5 ml chloroform: methanol (2:1) solution and the mixture was vortexed vigorously. The mixture was centrifuged for 2 min at 12,000 x g followed by the transfer of the bottom layer into a new Eppendorf microtube. The solution was air dried at room temperature and 1.5 ml cyclohexane was added and mixed vigorously. 1 ml of the solution was transferred into a quartz cuvette and absorbance was taken at 230 nm using GS-UV 32PCS spectrophotometer (General Scientific). CDs levels were calculated using the extinction coefficient of 29500 M^{-1} cm^{-1}. CDs levels were expressed as mmole/mg protein.

2.9. Determination of reduced glutathione

Reduced glutathione (GSH) in the tissue homogenate was assayed by the modified method of Asensi et al. (1999). Excised tissue was homogenised (x8 tissue weight) in 0.1 M sodium phosphate buffer, pH 7.5 (containing 1 mM EDTA). Equal volume of 20% trichloroacetic acid (TCA) was added to the homogenate and stand for 5 min at room temperature. The mixture was centrifuged at 2,000 *x g* for 10 min at 4 °C. 200 μl of the supernatant was added to 1.8 ml of 0.1 mM DNTB prepared in 0.1 M phosphate buffer, pH 7.5 and incubated for 5 min. Absorbance was measured at 412 nm using GS-UV 32PCS spectrophotometer (General Scientific). The GSH content of the sample was obtained by extrapolating the absorbance value of the sample from the standard GSH curve and results were expressed in mmole/mg protein.

2.10. Gene expression analysis by real time quantitative polymerase (RT-qPCR) reaction

Total RNA was extracted from the tissues using TRI Reagent® (Zymo Research, USA). 1 μg of RNA sample was used to synthesized cDNA by reverse transcriptase reaction using 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. Primers (Inqaba biotec, Hatfield, SA) to rat cDNA are listed in Table 1 and were used for PCR. Real time-quantitative PCR (qPCR) was performed using

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Table 1. Primer sequence for real time quantitative PCR.

Gene	Sequence
β-actin	Forward: CTCCCTGGAGAAGAGCTATGA Reverse: AGGAAGGAAGGCTGGAAGA
IL-8	Forward: GGGAGAAATCAGGGTGGATAAT Reverse: GGCAGCATCTGACAGAGTAAA
IL-10	Forward: TTGAACCACCCGGCATCTAC Reverse: CCAAGGAGTTGCTCCCGTTA
NF-κB	Forward: AGACATCCTTCCGCAAACTC Reverse: TAGGTCCATCCTGCCCATAA
IκB	Forward: CCACTCCATGTAGCTGTCATC Reverse: CACGTAGGCTCCGGTTTATT
IκKB	Forward: GAGAACAGGCCTTAGAGGATTT Reverse: CAATGATGTCACCTGAGCTTTC
JNK	Forward: ACGTGTCAAGAAACCTCCAATA Reverse: GATAACAGGGTGTCCGCTAAA
ERK1	Forward: GCCCTCCAATCTGCTTATCA Reverse: GCCACATACTCGGTCAGAAA
Nrf2	Forward: ACGTGATGAGGATGGGAAAC Reverse: TATCTGGCTTCTTGCTCTTGG
HO-1	Forward:GATGGCCTCCTTGTA CCATATC Reverse:AGCTCCTCAGGGAAGTAGAG
IL-1β	Forward: TCTGACAGGCAACCACTTAC Reverse: CATCCCATACACACGGACAA
TNFα	Forward: ACCTTATCTACTCCCAGGTTCT Reverse: GGCTGACTTTCTCCTGGTATG

Luna Universal qPCR Master Mix (BioLabs, New England) on a StepOnePlus Applied Biosystem qPCR System according to the manufacturer's protocols. PCR conditions were as follows: 95 °C for 3 min, 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Comparative cycle threshold (ΔΔCT) method was used to quantify the relative amount of cDNA. The β-actin gene was used to normalise the relative expression level of respective gene.

2.11. Statistical analyses

Statistical analysis was carried out using one-way analysis of variance (ANOVA) with Bonferroni post-hoc test for multiple comparisons between groups. GraphPad Prism5 Software was used for statistical analyses and data were expressed as mean ± SEM. Differences were considered statistically significant at the p-value of <0.05.

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3. Results

3.1. DEP methanol extract contains redox active compounds

The polycyclic hydrocarbons (PAHs) content of the DEP methanol extract was identified and quantified by GC-MS analysis and the results were shown in Table 2 and Fig. 1A. Fluoranthene and fluorene compounds are the most and least abundant of the PAHs, respectively, detected in the extract (Fig. 1A). The redox (oxidative) potential of the methanol DEP extract was evaluated using DTT as substrate (Fig. 1B-D). The result showed that DEP extract caused a decrease in DTT concentration with time compared to the blank (without DEP extract) (Fig. 1B). The rate of DTT loss with time was calculated using the slope of linear regression curves (Fig. 1C). The data showed that the extract caused about 0.35 μM DTT loss per min (Fig. 1D). This result indicates that the methanol DEP extract has redox active compounds that are capable of altering the redox status in cells.

3.2. Rooibos extract mitigate against DEP-induced oxidative damage

DEP extract caused significant increase ($p < 0.001$) in MDA and CDs levels in the aorta (Fig. 2A) and heart (Fig. 2B) when compared to control. However, rooibos extract pre-treatment caused a 1.99- and 1.26-fold significant reduction in MDA levels in the aorta ($p < 0.001$) and heart ($p < 0.01$), respectively, when compared

Table 2. PAHs composition in the methanol extract of DEP.

S/N	Target Compounds	R.T.	MW
1	Fluorene	10.497	166
2	2-methyl) Fluorene	10.723	181
3	1-methyl) Fluorene	10.753	181
4	Phenanthrene	12.763	178
5	Anthracene	15.344	178
6	Fluoranthene	15.715	202
7	2-methyl fluoroanthene	15.813	217
8	Pyrene	16.179	202
9	Benz[a]anthracene	19.223	228
10	Triphenylene	19.337	228
11	Benzo[e]pyrene	21.815	252
12	Benzo[b]fluoranthene	21.752	252
13	Benzo[a]pyrene	21.815	252
14	Indeno[1,2,3-cd]pyrene	24.212	278
15	Dibenz(a, h) anthracene	ND	ND

R.T: retention time; MW: molecular weight; ND: Not determine.

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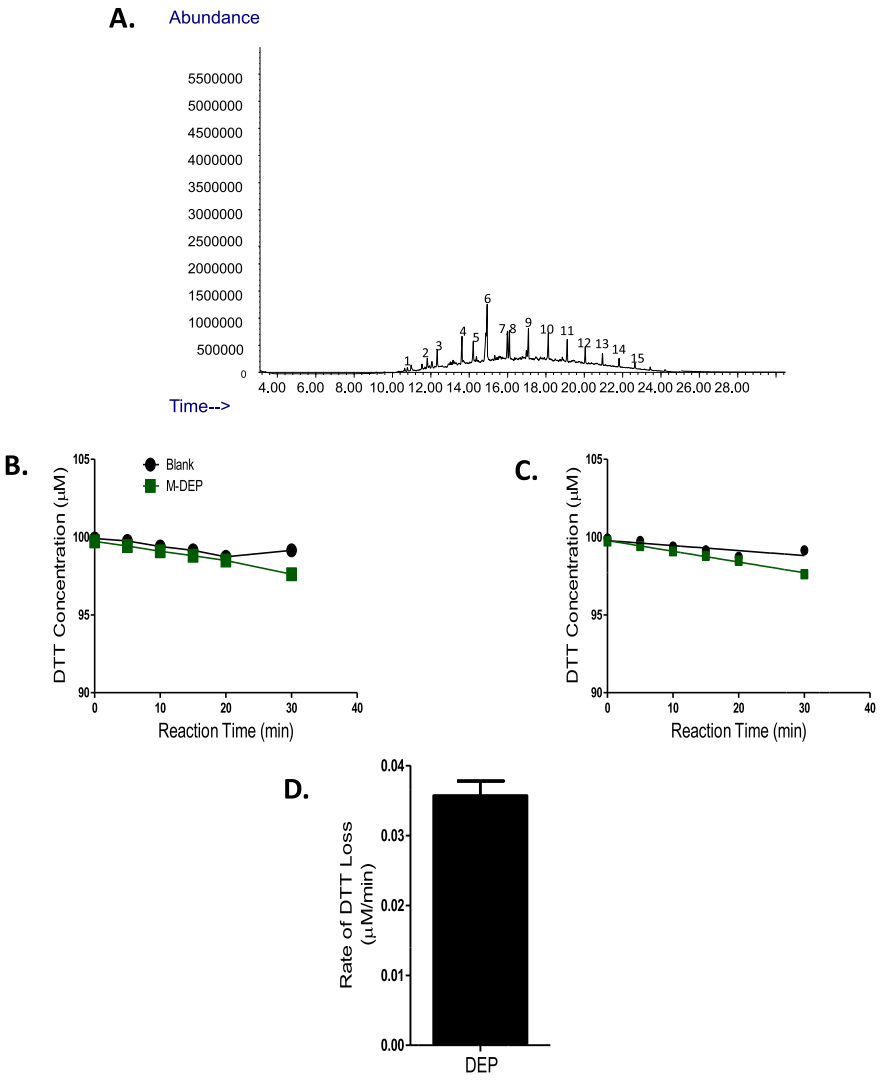

Fig. 1. GC-MS analysis and Redox potential of the constituents in DEP methanol extract. (A)The concentration versus time curve of PAHs eluent from GC-MS analysis of DEP extract. (B) Rate of DTT loss. The sample initially contains 100 μM DTT with DEP methanol extract. The blank initially contains 100 μM DTT with no extract. (C) Linear regression line and (D) Blank-corrected rate of DTT loss from DEP methanol extract. The rates of DTT loss for the extract and blank were determined as the slope of its sample regression line minus the blank slope. The rates of DTT loss are mean ± SEM of linear regression from triplicate (n = 3) experiments.

to DEP alone (Fig. 2). Also, the presence of rooibos extract significantly reduced CDs by 1.88- and 1.40-fold in aorta ($p < 0.001$) (Fig. 2A) and heart ($p < 0.01$) (Fig. 2B), respectively, when compared with rats exposed to DEP only. These results indicate the protective effects of rooibos extract on DEP-induced oxidative damage.

The GSH level in the aorta was significantly ($p < 0.05$) decreased by 0.7 mg/kg DEP compared to control (Fig. 2A). However, the presence of 50 mg/kg rooibos extract significantly ($p < 0.05$) mitigate against the reduction in aortic GSH level caused by

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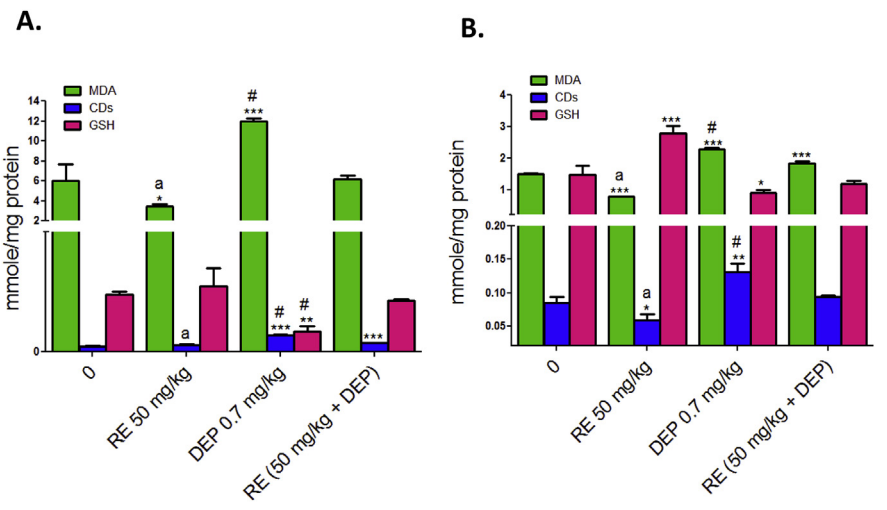

Fig. 2. Effect of rooibos extract on DEP-induced oxidative stress: MDA. CDs and GSH levels were determined in the (A) aorta and (B) heart of the exposed animals as described in the materials and methods. Values are mean ± SEM of six experiment animals done in triplicate (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 significant difference as compared to the controls. #p < 0.05 significant difference between RE pre-treated group and 10 μg/ml DEP. ap<0.05 significant difference between RE only and RE plus DEP.

DEP exposure (Fig. 2A). In the heart, exposure to 0.7 mg/kg DEP caused a 1.62-fold significant ($p < 0.05$) decrease in heart GSH level compared to control (Fig. 2B). Pre-treatment with rooibos extract does not have any significant effect on heart GSH level compared to DEP exposure only (Fig. 2B).

3.3. Rooibos extract modulates DEP-induced inflammation

The effects of rooibos extract on the DEP induction of inflammatory gene expression was assessed (Fig. 3). Exposure to 0.7 mg/kg DEP resulted in 1.71 and 1.36-fold significant ($p < 0.05$) increase in IL-8 gene expression in aorta (Fig. 3A) and heart (Fig. 3B), respectively, when compared to control. However, pre-treatment with rooibos extract caused a significant ($p < 0.001$) decrease in DEP-induced IL-8 gene expression in the aorta (1.44) and heart (5.49-fold) when compared to rat exposed to 0.7 mg/kg DEP alone (Fig. 3).

The results also showed that 0.7 mg/kg DEP exposure caused a 2.54 and 2.34- fold significant ($p < 0.001$) decrease in IL-10 mRNA in the aorta (Fig. 3A) and heart (Fig. 3B), respectively, when compared to control. However, while the presence of rooibos extract caused a 1.67- fold significant increase in IL-10 gene expression in the aorta compared to DEP alone (Fig. 3A), the pre-treatment produced 1.68-fold significant reduction in DEP-induced IL-10 mRNA level in the heart (Fig. 3B).

DEP caused 1.86-fold significant increase in TNFα gene expression in aorta when compared to control (Fig. 3A). However, pre-treatment with 50 mg/kg rooibos

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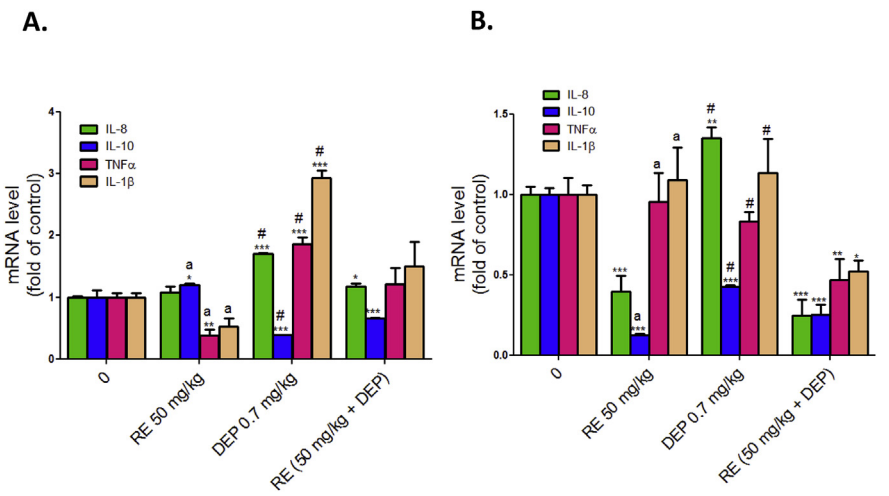

Fig. 3. Effect of rooibos extract on the inflammatory effect of DEP: The gene expressions of inflammatory cytokine-IL-8, IL-10, TNFα and IL-1β were determined in (A) aorta and (B) heart; by RT-qPCR using primers against the cDNA as described in the materials and methods. Values are mean ± SEM of six experiment animals done in triplicate (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001, as significant difference as compared to the controls. #p < 0.05 as significant difference between RE pre-treated group and 10 μg/ml DEP. [a]p<0.05 as significant difference between RE only and RE plus DEP.

extract caused a 1.53-fold significant ($p < 0.001$) reduction in TNFα mRNA levels induced by DEP in the aorta (Fig. 3A). No significant changes were seen in the heart TNFα mRNA levels between 0.7 mg/kg DEP and the control (Fig. 3B). In addition, rooibos extract pre-treatment caused a significant ($p < 0.01$) reduction in the heart TNFα gene expression induced by DEP (Fig. 3B).

Exposure to 0.7 mg/kg DEP caused a 2.92- fold significant ($p < 0.001$) increase in IL-1β mRNA levels in the aorta when compared to control (Fig. 3A). However, DEP exposure do not produced any significant change in IL-1β gene expression in the heart when compared to control (Fig. 3B). The IL-1β gene expression induced by DEP in the aorta and heart was significantly ($p < 0.001$) attenuated by 1.95 and 2.18-fold, respectively, in the presence of rooibos extract (Fig. 3).

3.4. Rooibos extract modulates DEP-induced NF-κB activation

We examined the modulatory effects of rooibos extract on the expression of genes in the NF-κB mediated inflammatory pathways in the aorta and heart of rats exposed to DEP (Fig. 4). Data showed that DEP caused 1.45 and 2.07-fold significant ($p < 0.01$) increase in NF-κB mRNA in the aorta (Fig. 4A) and heart (Fig. 4B), respectively, when compared to control. Pre-treatment with rooibos extract caused a 1.82 and 1.33- fold significant ($p < 0.01$) decrease in DEP-induced NF-κB gene expression in the aorta (Fig. 4A) and heart (Fig. 4B), respectively.

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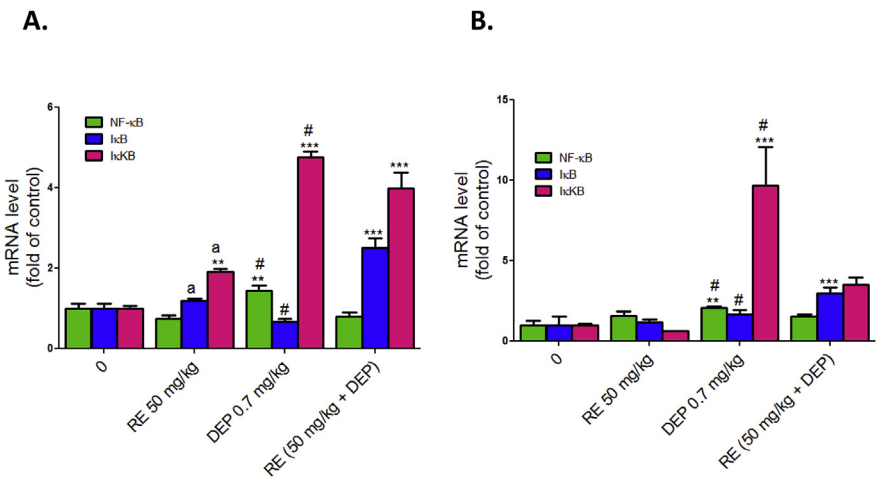

Fig. 4. Effect of rooibos extract on DEP induced NF-κB-mediated pathway: The mRNA levels of NF-κB, IκB and IκKB were determined in (A) aorta and (B) heart by RT-qPCR. Values are mean ± SEM of six experiment animals done in triplicate (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 as significant difference as compared to the controls. #p < 0.05 as significant difference between RE pre-treated group and 10 μg/ml DEP. ap<0.05 as significant difference between RE only and RE plus DEP.

Our results also showed that DEP exposure caused non-significant change in IκB gene expression in the aorta (Fig. 4A) and heart (Fig. 4B) when compared to control. Rooibos extract (50 mg/kg) pre-treatment, however, caused a 3.72 and 1.80-fold significant ($p < 0.001$) increase in IκB mRNA in the aorta and heart, respectively, when compared to rats exposed to 0.7 mg/kg DEP only (Fig. 4).

Also, exposure to DEP (0.7 mg/kg) caused 4.76 and 9.70- fold significant increase in IκKB (IκB kinase) mRNA levels in the aorta (Fig. 4A) and heart (Fig. 4B), respectively, when compared to control. The presence of 50 mg/kg rooibos extract, however, significantly ($p < 0.01$) attenuated DEP-induced IκKβ gene expression in the aorta and heart by 1.19 and 2.75-fold, respectively. These data indicate that NF-κB/IκB/IκKB pathway may be involved in the DEP-induced inflammatory response and the anti-inflammatory effect of rooibos extract on DEP-induced inflammation may be exerted via this pathway.

3.5. Rooibos extract modulates the gene expression of redox signalling molecules in the presence of DEP

Our data showed that DEP exposure caused significant ($p < 0.001$) decrease in ERK1 gene expression in the aorta when compared to control (Fig. 5A). In contrast, DEP caused significant ($p < 0.01$) increase in heart ERK1 gene expression when compared to control (Fig. 5B). Pre-treatment with rooibos extract (50 mg/kg), however, significantly ($p < 0.05$) attenuated DEP-induced ERK1 mRNA in the aorta (2.97-fold) and heart (1.29-fold) (Fig. 5).

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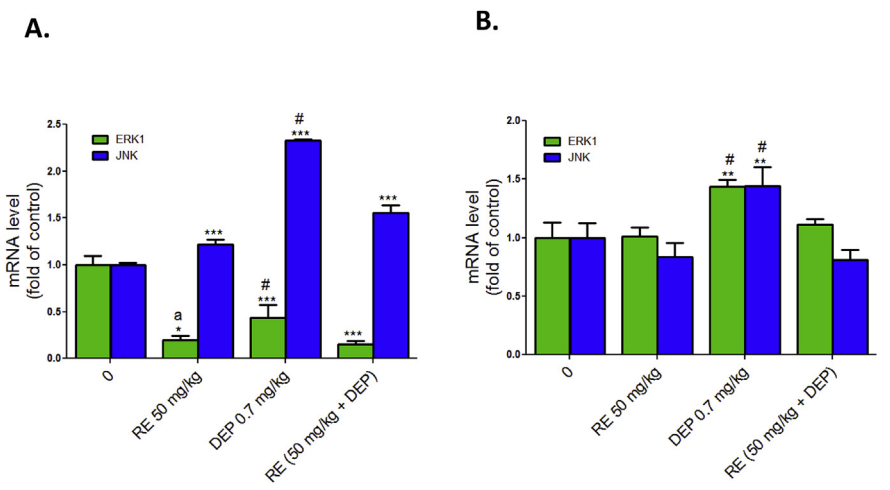

Fig. 5. Effect of rooibos extract on the redox signalling effect of DEP. ERK1 and JNK mRNA levels were quantified in (A) aorta and (B) heart by RT-qPCR. Values are mean ± SEM of six experiment animals done in triplicate (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 as significant difference as compared to the controls. #p < 0.05 as significant difference between RE pre-treated group and 10 μg/ml DEP. [a]p<0.05 as significant difference between RE only and RE plus DEP.

Our results showed that exposure to 0.7 mg/kg DEP caused 2.33- and 1−44-fold significant ($p < 0.001$) increase in JNK gene expression in the aorta (Fig. 5A) and heart (Fig. 5B), respectively, when compared to control. However, 50 mg/kg rooibos extract pre-treatments significantly ($p < 0.001$) decreased DEP-induced JNK gene expression in the aorta and heart by 1.49- and 1.78- fold, respectively (Fig. 5). These data indicate that ERK1 and JNK signalling molecules activation may play a role in DEP-induced inflammation in the cardiovascular system of exposed rats. In addition, these signalling molecules may be an important target in the modulating effect of rooibos extract on DEP-induced cardiovascular inflammation.

3.6. Rooibos extract attenuates DEP induction of Nrf2 and Nrf2-dependent gene expression

The present work shows that exposure to 0.7 mg/kg DEP caused a 2.05- and 1.98-fold significant ($p < 0.01$) increase in Nrf2 mRNA level in the aorta (Fig. 6A) and heart (Fig. 6B), respectively, when compared to control. However, pre-treatment with 50 mg/kg rooibos extract caused a 1.98- and 2.00- fold significant ($p < 0.001$) reduction in DEP-induced Nrf2 gene expression in the aorta and heart respectively (Fig. 6).

The expressions of HO-1 gene in the aorta (2.29-fold) (Fig. 6A) and heart (1.66-fold) (Fig. 6B) were significantly ($p < 0.001$) increased by 0.7 mg/kg DEP when compared to control. In contrast, pre-treatment with 50 mg/kg rooibos extract caused a 2.47- and 1-88- fold significant decrease in DEP-induced HO-1 mRNA level in the aorta and heart, respectively (Fig. 6). These data suggest the antioxidant ability of the

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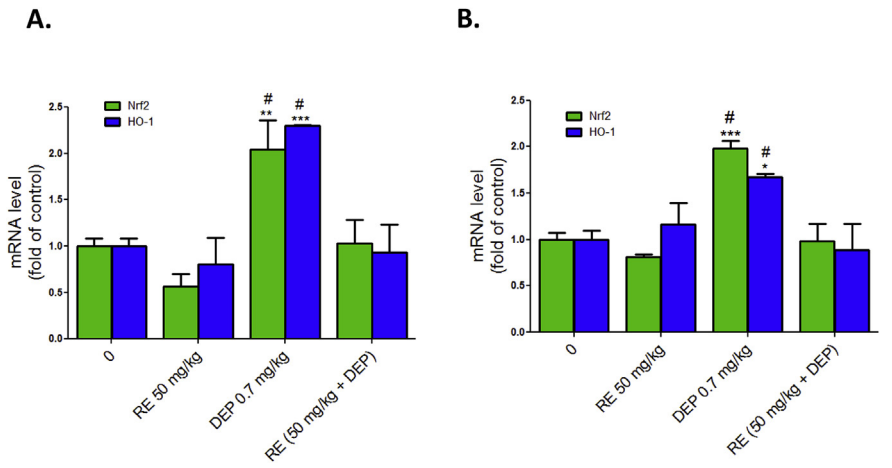

Fig. 6. Effect of rooibos extract on DEP-induced Nrf2-mediated antioxidant response. Nrf2 and HO-1 mRNA levels were determined in (A) aorta and (B) heart by RT-qPCR. Values are mean ± SEM of six experiment animals done in triplicate (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 as significant difference as compared to the controls. #p < 0.05 as significant difference between RE pre-treated group and 10 μg/ml DEP. [a]p<0.05 as significant difference between RE only and RE plus DEP.

rooibos extract in protecting against the DEP-induced oxidative stress without triggering the endogenous antioxidant enzymes production.

4. Discussion

Our data show that aqueous rooibos extract exerts antioxidant and anti-inflammatory effects on diesel exhaust particles induced oxidative stress and inflammation in the aorta and heart of exposed wistar rats. This is the first study showing the cardiovascular protective effects of rooibos on diesel exhaust particles in rat.

DEP contained redox active compounds such as PAHs, metals and electrophiles (Lawal et al., 2016). These compounds have been shown in several studies to exert their damage in a mechanism that involves induction of oxidative stress and inflammation (Robertson et al., 2012; Miller et al., 2013; Lawal et al., 2015). The ability of these DEP chemical constituents to exert oxidative stress depends on their redox capacity, which alters the redox balance in the cells leading to alteration in the activity and expression of redox signalling molecules such as ERK1, MAPK, JNK etc. In this study, DEP methanol extract exhibited a redox capacity as it caused a loss of DTT concentration with time. DTT is a thiol containing compounds that can be oxidized by the presence of oxidant or redox active compounds. The results showed that DEP caused 0.035 μM/min reductions in DTT concentration indicating the presence of redox active compounds in the DEP extract from sample used in this study. This result supports the work earlier done that showed that chemical compounds in a particulate matter (such as PAHs, metals) is redox active with varying oxidative potential (Charrier and Anastasio, 2012). Though the assay was done in the dark, auto

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oxidation of DTT may still occur to certain level and this could account for the small difference in the level of DTT oxidation between the DEP sample and the control. In addition, the oxidative potential of the PAHs in the DEP extract may account for only a small fraction of the total oxidative capacity of the DEP. Furthermore, the in vitro oxidative effect of DEP extract may be lower compared to its *in vivo* oxidative effect likely due to the presence of more targets macromolecules that are prone to DEP oxidation.

The presence of redox active PAHs was confirmed in our GC-MS data. Our data showed that the DEP used in this study contains varying amount of different PAHs with fluorathene and fluorene as the most and least abundant PAHs, respectively. Our results were in agreement with the work of Schuetze (1983), which showed the presence of PAHs of varying polarities and concentrations in diesel exhaust particles from a motor vehicle (Lawal et al., 2016).

Different studies, using animal models, have used varying concentrations of DEP based primarily on the route of administration (Nikula et al., 2001; Danielsen et al., 2008). In our previous in vitro study, we exposed human microvascular cells (HMEC) to 5−50 μg/ml DEP in a cell density of 2.0×10^5 cells/ml corresponding to 5−50 μg/million cells assuming uniform distribution (Lawal et al., 2015). The dose of DEP used in this present study falls likely within the concentrations range of realistic exposure to air pollutants that eventually become bio-available. This dose corresponds to 0.7 μg DEP/g body weight containing millions cells. This dosage is likely to enhance the systemic translocation of DEP to the internal organs such as the lung and liver. Furthermore, the DEP administered subcutaneously needs to pass through the epidermal skin layer and internalized in the keratinocytes to cause effects or engulfed by the monocyte-derived macrophage to eliminate effects. Either of the two scenarios reduced the bio-availabilty of the subcutaneously administered DEP in the systemic circulation and the corresponding amount that eventually gets into the tissues.

DEP induction of oxidative stress and its resultant cellular impacts are well established in several studies (Lawal et al., 2016). The induction of reactive oxygen species (ROS) production by DEP overwhelms the antioxidant defense with depletion of GSH and accumulation of GSSG leading to oxidative damage to macromolecules (Chirino et al., 2010; Tobwala et al., 2013; Forchammer et al., 2012; Lawal et al., 2016; Lawal, 2017). Our present study agrees with these previous findings. We found significant increase in oxidative biomarkers-MDA, CDs and decrease in GSH levels in the aorta and heart of animals exposed to diesel exhaust particles extract. Our data also showed that DEP caused induction in Nrf2 and HO-1 gene expression probably in response to oxidative stress. This was in confirmation with our earlier work and that of others, where up-regulation in antioxidant

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genes expression was observed in cells and animals exposed to DEP (Lawal et al., 2015; Miller et al., 2013; Davel et al., 2012).

The ability of DEP to induce inflammation is in direct proportion to their ability to induce oxidative stress, which correlates with their redox potential (Lawal, 2017; Lawal et al., 2016; Charrier and Anastasio, 2012). In this study, DEP increased the expression of pro-inflammatory genes; IL-8, TNFα and IL-1β, while decreasing the expression of anti-inflammatory IL-10 gene. This further supports the pro-inflammatory activities of diesel exhaust, which has been well established in many in vitro and in vivo studies (Lawal et al., 2015, 2016; Miller et al., 2013; Robertson et al., 2012). The oxidative stress induced by DEP leads to activation of redox-sensitive signalling pathways such as the MAPK kinases, ERK1, JNK and the NF-κB cascade with the consequent activation of cytokines and chemokines (Lawal et al., 2016; Lawal, 2017; Lee et al., 2012; Montiel-Davalos et al., 2010). In line with these previous findings, our present study revealed that diesel exhaust particles caused significant elevation in the gene expression of the signalling molecules-ERK1, JNK, NF-κB and IκKB probably in response to increase oxidative stress leading to increase expression of inflammatory genes.

The involvement of NF-κB in diesel exhaust particles induced inflammation was also highlighted in this present study. NF-κB mediated pathways regulate pro-inflammatory pathways and inflammatory cytokines production (Monaco et al., 2004). Two separate pathways can activate NF-κB cascade: the canonical and the alternate pathways. TNFα, Toll-like microbial pattern recognition receptors (TLRs) and IL-1 activate the canonical pathway, while the alternate pathway is activated by TNF-family cytokines (with exception of TNFα) (Karin and Ben-Neviah, 2000; Bonizzi et al., 2004). The presence of TNFα or IL-1 activates the inhibitory kappa B kinase (IκKB), which phosphorylate inhibitory kappa B (IκB) with the consequent release and activation of NF-κB (Lawrence, 2009). In this study, diesel exhaust particles caused the increase gene expression of TNFα, IL-1β, IκKB and NF-κB, but decrease IκB gene expression thereby promoting NF-κB activation and nuclear migration. This data suggest that the pro-inflammatory effect of DEP is likely to be exerted via NF-KB mediated pathways in agreement with earlier studies (Lawal, 2017).

The use of phytochemicals in disease treatment and prevention has been well established in different studies (Marnewick et al., 2009, 2011). In the present study, we found that aqueous extract of rooibos mitigates against the oxidative and pro-inflammatory effects of DEP in the aorta and heart of exposed rats. The extract protects against lipid peroxidation (as measured by MDA and CDs levels) and GSH depletion induced by DEP. Our results were consistent with earlier experimental and clinical studies that have reported on the antioxidant potential of the extract (Marnewick et al., 2011). Rooibos contains many phenolic compounds with antioxidant property,

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which can interfere with the free radical chain reaction without activating the Nrf2-dependent pathway. This was confirmed in our results, which revealed the protective effect of the extract without induction of Nrf2-regulated enzyme. Rooibos was also found to suppress the inflammatory effect of DEP by modulating the TNFα or IL-1β/IκKB/IκB/NF-κB pathways. Thus, confirming the anti-inflammatory effect of rooibos in agreement with earlier studies, which found that rooibos and flavonoids exhibit anti-inflammatory effects (Hendricks and Pool, 2010; Steptoe et al., 2007). Rooibos also suppressed the diesel exhaust particles activation of redox sensitive signalling ERK1 and JNK molecules, both of which have been implicated in inflammation, apoptosis, cell cycle and immune response (McCubrey et al., 2007; Trachootham et al., 2008). The rooibos extract dose of 50 mg/kg used in this study is achievable by oral administration in humans as shown in a clinical study by Marnewick and co-workers (Marnewick et al., 2011). The study reported that oral consumption of 6 cups of rooibos tea (a cup contains one tea bag in 200 ml boiled water) per day for 6 weeks does not have any adverse health effects on human participants. In addition, a tea bag weighs ~2.5 g implying that approximately 15 g of tea leaves prepared in 1.2 L boiled water were consumed daily for 6 weeks (i.e. 630 g tea leaves).

In summary, this study demonstrates that exposure of rats to DEP caused significant oxidative stress and pro-inflammatory response in the cardiovascular system by modulating the Nrf2- and NF-κB regulated pathways. The reduction in air pollution, through proper monitoring by various regulatory agencies, is the key strategy to reducing the health effects of air particulate matters. However, the consumption of herbal products, as exemplified by rooibos tea, could help to attenuate the harmful effects of air particulate matter and thus prolong lifespan.

Declarations

Author contribution statement

Akeem O. Lawal: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Dare M. Oluyede, Monsurat O. Adebimpe, Lateefat T. Olumegbon, Olamide O. Awolaja: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Olusola O. Elekofehinti, Olamide O. Crown: Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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