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The effects of Rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia species*) on air pollutant chemicals-induced cytoxicity in human skin fibroblasts and keratinocytes

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Abstract

Diesel Exhaust Particles (DEP) have been reported to be mainly responsible for most of the toxic effects of air pollution probably via a mechanism that involves induction of oxidative stress and proinflammatory response. Rooibos (*Aspalathus linearis*) and Honeybush (*Cyclopia species*) are widely used due to their health promoting effects. This study examined the effect of Rooibos tea and Honeybush on the prooxidative and proinflammatory effects of standard reference material (SRM) 2975 DEP in human skin fibroblasts and immortalized keratinocyte cell lines. The cells were either exposed to different concentrations (5-50 μg/ml) of DEP for 4 h or pre-treated with 40 and 60 μg/ml of fermented (FR) or green (GR) Rooibos or fermented Honeybush (FH) for 6 h prior to 4 h exposure to 10 μg/ml DEP. Exposure to DEP caused a significant decrease in cell viability, and increase in LDH leakage, ROS production, oxidative stress biomarkers and pro-inflammatory cytokines in both cell lines compared to control. However, the presence of the extracts significantly ($p < 0.001$) attenuate these effects. This study highlights the potential of FR, GR and FH in protecting against DEP-induced oxidative stress and proinflammatory effect in fibroblasts and keratinocytes probably.

Keywords: *Fibroblasts, Keratinocytes, Diesel Exhaust Particles (DEP), Rooibos extracts, Honey bush*

1. Introduction

Several epidemiological studies have shown an association between environmental pollution and various health hazards (Kelly and Fussell, 2015). Diesel exhaust particles (DEP) are important and major component of air particulate matter in the environment, which contribute significantly to the adverse human health effects of air pollution (Lawal *et al.*, 2015; and Lawal *et al.*, 2016). As an environmental pollutant, DEP is capable of interacting directly with the epidermal skin layer, which is the largest organ in the body, that forms

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an interface between the internal and external environment. Studies have shown that DEP can become internalized by keratinocytes and thus promote pro-oxidative, profibrotic and pro-inflammatory responses in normal human skin (Fiorito *et al*., 2011; and Mastrofrancesco *et al*., 2014)

The epidermal cells form the outermost barrier in direct contact with environmental air pollutants and represent the largest population of cells that is directly exposed to chemical pollutants such as DEP. The exposure of these cells to the chemical pollutants can trigger their activation with the consequent release of pro-oxidant and pro-inflammatory cytokines, which in turn can activate other cells, such as fibroblasts, to produce molecules that are fibrogenics (Simijs and Bouwstra, 2010; and Vierkotter and Krutmann, 2012). The adverse effects of DEP on human health are currently of great concern since it can induce various types of cancers, and cardiovascular diseases (Silverman, 2017; and Neophytou *et al*., 2019). In order to respond to and function efficiently to environmental hazard, such as air pollutant, the skin is equipped with the ability to regenerate in a carefully regulated manner (Lim and Nusse, 2013). Disruption to this regulatory process results in skin diseases such as cancers.

The Wnt signaling pathway plays an important role in skin morphogenesis and organogenesis (Widelitz, 2008) The canonical Wnt pathway is activated by the translocation of â-catenin to the nucleus leading to expression of Wnt target genes (Vikram *et al*., 2014; and Yang *et al*., 2016). Wnt/β-catenin signaling is regulated by phosphorylation/desphosphorylation mechanism. While β-catenin desphosphorylation is require for canonical Wnt signaling activity, phosphorylation of β-catenin by a multiple protein complex including glycogen synthase kinase- 3β (GSK-3β) results in its ubiquitin-mediated degradation (Gao *et al*., 2014).

Rooibos (*Aspalathus linearis (Burm.f.) R.Dahlgren*) and Honeybush (*Cyclopia species*), are medicinal plants that are widely used due to their health promoting effects (Marnewick *et al*., 2011; and Mahomoodally, 2013). Studies have shown that they contain important flavonoids with powerful antioxidant and anti-inflammatory capacities (Lawal *et al*., 2019a; and Mahomoodally, 2013). These herbal teas have been shown to exhibit anti mutagenic, antioxidant, cancer modulating, and cardiovascular activities (Lawal *et al*., 2019b; and Hong *et al*., 2014).

In this present study, we aimed to investigate the effects of Rooibos and Honeybush against the pro-oxidative and pro-inflammatory effects of environmental air pollutants, such as DEP, in human skin keratinocytes and fibroblasts in order to provide an alternative therapy for the treatment of skin diseases induced by environmental pollutants.

2. Materials and methods

2.1. Chemicals

DEP (standard reference material 2975, SRM 2975) was obtained from National Institute of Standards and Technology (NIST, Gaithersburg, MD). Dulbecco Modified Eagle Medium (DMEM), fetal calf serum (FCS) and penicillin-streptomycin (Pen/Strep) were purchased from Lonza (Whitehead Scientific, SA). In Vitro Toxicology Assay Kit, Lactic Dehydrogenase based (TOX7) was purchased from Sigma-Aldrich (Sigma-Aldrich, SA). 2′, 7′-dichlorofluorescein diacetate (DCF-DA) and protein carbonyl content assay kit (MAK094) were purchased from Sigma-Aldrich (South Africa). Pierce™BCA Protein Assay Kit (23227) was obtained from Thermo Scientific (Fairland, SA). M-MLV Reverse Transcriptase was obtained from Promega (Madison, WI). PowerUp™SYBR® Green Master Mix (A25742) was purchased from Applied Biosystems (Austin, TX). alamarBlue® Cell Viability Reagent (DAL1025) was obtained from Invitrogen (Fairland, SA). All other chemicals and reagents are of the highest grade and were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Rooibos and honey bush aqueous extracts preparation

Both fermented rooibos (FR) and green rooibos (GR), and fermented Honeybush (FH) powders were generous gifts from Rooibos Limited (Clanwilliam, South Africa) and have been well characterized in our previous study (Lawal *et al*., 2019a). The extracts were prepared by reconstituting the lyophilized samples in double distilled water (10 mg/ml) at room temperature as previously described (Lawal *et al*., 2019a).

2.3. DEP organic extracts preparation

A well characterized DEP, SRM 2975, was obtained from NIST and has been widely used in many comparison studies with other types of DEPs (Forchhammer *et al*., 2012; and Lawal *et al*., 2015). The methanolic extract of DEP (SRM 2975) was carried out as previously described (Lawal *et al*., 2015).

2.4. Cell culture and treatment

Primary human fibroblast obtained from the neonatal foreskin and human foreskin immortalized keratinocyte were cultured in 100 mm cell culture dish (Whitehead Scientific) in DMEM supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (Pen/Strep). The cells were maintained at 37°C in a humidified atmosphere at 5% CO_2 and 95% air. Treatments were done in appropriate cell culture plate. Cells were seeded at a density of 10^5 cells/ml for treatment in 100 mm cell culture dish in a total volume of 8 ml/dish. Cells were treated with 40 and 60 μg/ml of either FR or GR or FH, prepared from the stock (10 mg/ml) in growth media, for 6 h prior to treatment with 10 μg/ml DEP for 4 h at 37°C. After the incubation period, the cells were harvested in PBS and used for various assays.

2.5. Intracellular reactive oxygen species production

Dichlorofluoresscin diacetate (DCF-DA) method was used to monitor ROS production by DEP as previously described (Lawal *et al*., 2019a). Fluorescence intensity was monitored every 15 min for 2 h after exposure to DEP (5-50 μg/ml) and 30 and 60 min in a pre-treatment experiment. Fluorescence was taken at 485 nm excitation and 530 nm emission using Cary Eclipse Fluorescence Spectrophotometer (Varian, SA).

2.6. Cell lysate preparation and protein determination

After the treatment of cells with appropriate agents, the cells were washed and scraped into an Eppendorf microtube in PBS. The cell lysate was prepared using RIPA extraction buffer according to the manufacturer protocol. Pierce™BCA protein assay kit (Thermo Scientific; Fairland, SA) was used to determine the protein content in the cell lysates.

2.7. Determination of oxidative stress biomarkers

Protein carbonyl content assay kit (Sigma-Aldrich, SA) was used to determine the protein carbonyl level in the cell lysate according to the manufacturer's protocol (Purdel *et al*., 2014). The amount of the conjugated dienes formed in the cell lysate was determined as previously described (Lawal *et al*., 2019b). Thiobarbituric acid (TBA) method was used to determine the level of lipid peroxidation in the cell lysate (De Leon and Borges, 2020). The GSH/GSSG ratio was determined as previously described (Owen and Butterfield, 2010).

2.8. Cytotoxicity assays

AlamarBlue® Cell Viability Reagent kit (Invitrogen) was used to measure the population of surviving cells after DEP exposure in the absence or presence of either FR, or GR, or FH as previously described (Lawal *et al*., 2019a) The In Vitro Toxicology Assay Kit, Lactic Dehydrogenase Based (Sigma-Aldrich, SA) was used to assess lactic dehydrogenase (LDH) leakage as an indices of cell membrane damage as previously described (Lawal *et al*., 2019a).

2.9. ImmunoCytoChemistry

Cells were seeded on a coverslip placed inside a 2 ml culture dish at a density of 2×10^6 cells/ml and allow to attach for 24 h. Cells were then treated with 5-50 μ/ml DEP for 4 h and after which the dish was washed with PBS. Nrf2 localization in the cells were detected using Rabbit polyclonal Nrf2 antibody (1:50) and Goat anti Rabbit Alexa 488 (1:300) according to manufacturer protocols. Visualization was carried out with a fluorescent microscope.

2.10. Gene expression

RNeasy® Mini Kit (Qiagen) was used to extract total RNA after cell treatment and M-MLV Reverse Transcriptase (Promega) was used to synthesize cDNA from 1μg RNA sample by a reverse transcriptase method in a 2-steps

reaction conditions: 70°C for 5 min and 42°C for 1 h. Primers (integrated DNA technology) to human cDNA are listed in Table 1. The real-time quantitative PCR (qPCR) was carried out using the StepOnePlus Real-time PCR system (Applied Biosystems) according to the manufacturer's protocols. PCR conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, 95°C for 15 s and 60°C for 1 min. Comparative cycle threshold method ($\Delta\Delta$CT method) was used to quantify the relative amount of cDNA as described by Applied Biosystems. The Cp values were normalized to the respective Cp values of β-actin reference gene.

Table 1: Primer sequence for real-time quantitative PCR

Genes	Sequences
β-actin	Forward: CCAACCGCGAGAAGATGACC Reverse: GATCTTCATGAGGTAGTCAGT
IL-6	Forward: AGGCACTGGCAGAAAACAAC Reverse: TTTTCACCAGGCAAGTCT
IL-8	Forward: TGTCAGCACAACATGGTCTTG Reverse: TGAGGTTTTGATTGTTCATGTCTT
TNFα	Forward: AACCTCCTCTCTGCCATC Reverse: ATGTTCGTCCTCCTCACA
ATF4	Forward: TCTCCAGCGACAAGGCTAA Reverse: CAATCTGTCCCGGAGAAGG
NQO1	Forward: ATGTATGACAAAGGACCCTTCC Reverse: TCCCTTGCAGAGAGTACATGG
CYP1B1	Forward: TGATGGACGCCTTTATCCTC Reverse: CCACGACCTGATCCAATTCT

Note: IL – Interleukin, TNFα – Tumor necrotic factor alpha, ATF4 – Activated transcription factor-4, NNQO1 – NADPH quinone oxidoreductase 1, CYP1B1 – Cytochrome P450 1B1.

2.11. Western blot analysis

The whole cell extracts were used for western blot after treatment total protein (30 μg) was separated by SDS-PAGE and transferred to nitrocellulose membrane (Hybond ECL) for 2 h at 200 mA. Phospho-β-catenin, GSK-3β and Wnt3 proteins were detected using rabbit monoclonal anti-phospho-β-cateinin, anti-GSK-3β and anti-Wnt3 antibodies (1:1000) respectively and goat anti-rabbit antibody (1:2000) conjugated to HRP according to the manufacturer's protocol. β-actin protein was detected by anti-β-actin antibody (1:2000) and used for the normalization of protein loading. ECL reagent was used to develop the blots according to the manufacturer's protocol.

2.12. Statistical analyses

All data were expressed as mean ± SEM. GraphPad Prism5 Software was used for statistical analyses and analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni post-hoc test for multiple comparisons between groups. Statistically significant differences between data were taken at the p-value of <0.05.

3. Results

3.1. DEP-induced ROS production was attenuated by Rooibos and Honeybush extracts

Our data show a time-dependent increased in ROS production in fibroblasts exposed to 5-50 μg/ml DEP (Figure 1A). ROS production in fibroblasts was significantly ($p < 0.001$) elevated at all the DEP concentrations and time compared to control (Figure 1A). Pre-treatment with 40 and 60 μg/ml of either FR, GR or FH for 6 h caused a significant ($p < 0.05$) reduction in ROS production, except at 40 μg/ml HB, after 30 min exposure to DEP in fibroblasts (Figure 1B). Also, exposure to 5-50 μg/ml DEP for 15 to 120 min also caused a time dependent significant ($p < 0.001$) increased in ROS production in keratinocytes compared to control (Figure 1C). However, pre-treatment of keratinocytes with 40 and 60 μg/ml of either FR, GR and FH for 6 h caused a significant ($p < 0.001$) decrease in DEP-induced ROS production at 30 min exposure (Figure 1D). Both GR and FH also significantly decreased ROS production in keratinocytes at 60 min exposure to DEP (Figure 1D).

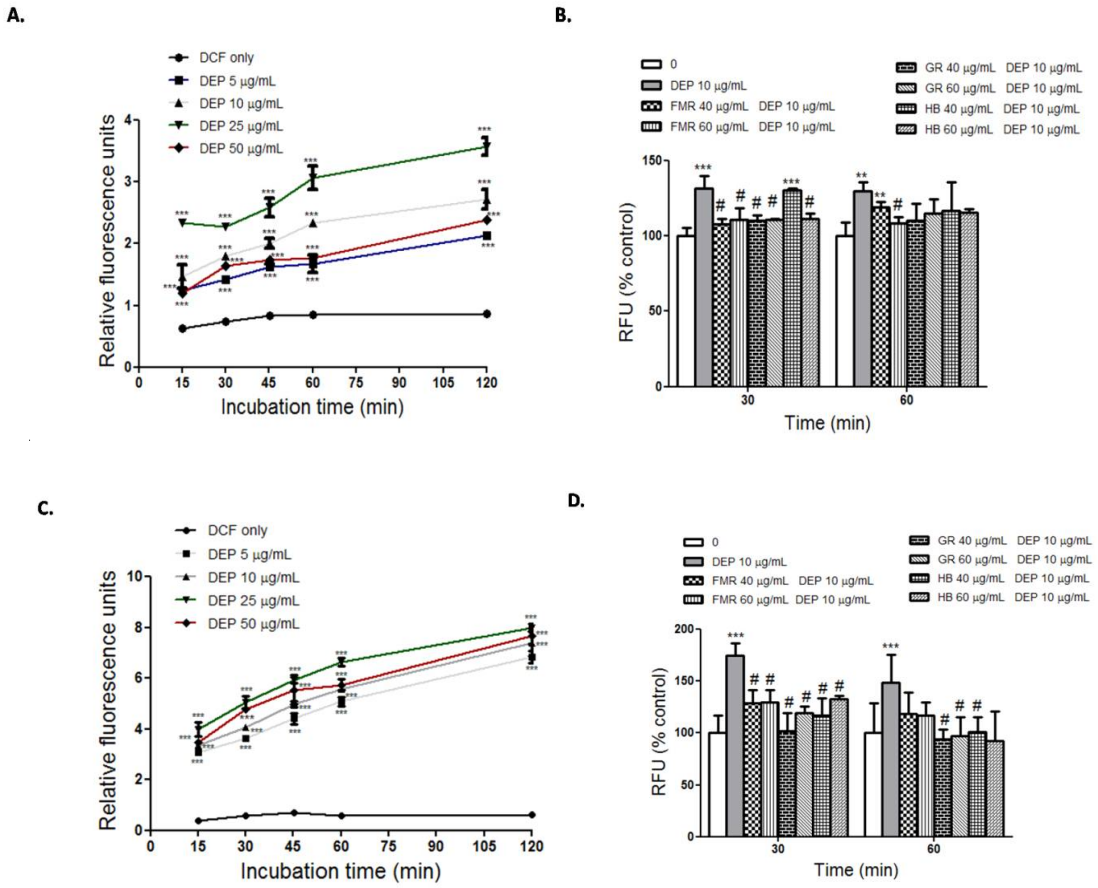

Figure 1: Effect of aqueous extracts of rooibos and honeybush on DEP-induced ROS production. ROS levels were determined in (A,B) fibroblasts and (C, D) keratinocytes as described in the materials and methods. The values shown are mean ± SEM of three different experiments done in triplicate ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as significantly different when compared with the control cells and $^{\#}p < 0.01$ as significantly different when compared with 10 μg/ml DEP-exposed cells.

3.2. Rooibos and Honeybush extracts offer protection against DEP-induced oxidative damage

Our results show that exposure to 10 and 25 μg/ml DEP caused significant ($p < 0.05$) increase in protein carbonyl contents, conjugated dienes production and MDA levels in fibroblasts and keratinocytes when compared to control (Table 2). Pre-treatment of these cell lines with 40 and 60 μg/ml of either FR, GR or FH prior to 10 μg/ml DEP exposure, in most cases, resulted in significant ($p < 0.01$) decrease in the levels of these oxidative markers in both cell lines (Table 2).

In contrast, 10 and 25 μg/ml DEP exposure caused significant ($p < 0.001$) decrease in GSH/GSSG ratio in fibroblast and keratinocytes when compared to control (Table 2). However, pre-treatment of both cell lines with 40 and 60 μg/ml of either FR, GR or FH before exposure to 10 μg/ml DEP significantly ($p < 0.001$) increased the GSH / GSSG ratio when compared with cells exposed to 10 μg/ml DEP only (Table 2).

Table 2 Effects of DEP on oxidative and redox status in fibroblasts and keratinocytes pre-treated with the herbal tea extracts

Treatment	Biomarkers							
	CPs (nmol/mg)		CDs (μmol/mg)		MDA (nmol/mg)		GSH:GSSG	
	Fibroblasts	Keratinocytes	Fibroblasts	Keratinocytes	Fibroblasts	Keratinocytes	Fibroblasts	Keratinocytes
Negative ControlDEP (10 μg/ml)	43.04± 0.21 47.99± 1.73***	16.21± 0.55 27.08± 0.55***	8.50± 0.27 9.42± 0.13*	4.57± 0.12 9.19± 0.29***	0.26± 0.05 0.42± 0.03**	0.27± 0.001 0.35± 0.001*	3.02± 0.02 0.51± 0.02***	1.88± 0.01 0.52± 0.01***
Fermented Rooibos (FR): 40 μg/ml + DEP60 μg/ml +DEP	40.03± 0.09$^{**,\#}$ 31.69± 0.03$^{***,\#}$	30.69± 1.04$^{***,\#}$ 16.69± 0.07$^{\#}$	9.53± 0.15* 8.55± 0.45	4.30± 0.31$^{\#}$ 6.60± 0.41$^{***,\#}$	0.30± 0.02$^{\#}$ 0.28± 0.05$^{\#}$	0.33± 0.02 0.27± 0.03$^{\#}$	1.60± 0.02$^{***,\#}$ 2.02± 0.02$^{***,\#}$	0.65± 0.01$^{***,\#}$ 1.09± 0.01$^{***,\#}$
Green Rooibos (GR): 40 μg/ml + DEP60 μg/ml + DEP	40.15± 0.18$^{**,\#}$ 27.83± 0.10$^{***,\#}$	18.45± 0.18$^{***,\#}$ 17.61± 0.11$^{**,\#}$	7.54± 0.35$^{\#}$ 9.27± 0.28	6.66± 0.46$^{***,\#}$ 5.88± 0.34$^{**,\#}$	0.28± 0.02$^{\#}$ 0.31± 0.03$^{\#}$	0.28± 0.02$^{\#}$ 0.34± 0.04*	1.20± 0.03$^{***,\#}$ 3.02± 0.03$^{\#}$	0.87± 0.01$^{***,\#}$ 0.92± 0.02$^{***,\#}$
Fermented Honeybush (FH): 40 μg/ml + DEP60 μg/ml + DEP	58.30± 0.34$^{***,\#}$ 47.97± 0.55***	18.32± 0.10$^{***,\#}$ 25.04± 0.26$^{***,\#}$	10.29± 0.33*** 8.15± 0.47$^{\#}$	5.67± 0.32$^{*,\#}$ 5.82± 0.33$^{**,\#}$	0.36± 0.08 0.38± 0.02	0.22± 0.03$^{\#}$ 0.21± 0.03$^{\#}$	0.60± 0.04$^{***,\#}$ 1.89± 0.04$^{***,\#}$	1.17± 0.02$^{***,\#}$ 1.38± 0.01$^{***,\#}$

Note: Values are mean ± SD of three ($n = 3$) different experiments done in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ are significantly different when compared to control, with $^{\#}p < 0.01$ significantly different when compared to the 10 μg/ml DEP control.

3.3. DEP-induced cytotoxic effects was attenuated by Rooibos and Honeybush extracts

There was a significant ($p < 0.01$) decreased in fibroblast cell viability exposed to 5-50 μg/ml DEP for 4 h (Figure 2A). Pre-treatment of fibroblast with 60 μg/ml FR, 40 and 60 μg/ml FH caused a significant ($p < 0.01$) increase in viability when compared to cells exposed to 10 μg/ml DEP only (Figure 2B). DEP (5-50 μg/ml) also caused significant decrease ($p < 0.01$) in the viability of keratinocytes when compared to control (Figure 2C). Pre-treatment of keratinocytes with 40 and 60 μg/ml of either FR, GR or FH resulted in significant ($p < 0.001$) increase in viability when compared to cells treated with 10 μg/ml DEP only (Figure 2D).

Exposure to 5-50 μg/ml DEP caused significant ($p < 0.001$) increase in LDH leakage in fibroblasts when compared to control (Figure 3A). However, pre-treatment of fibroblast with 40 and 60 μg/ml FR, GR and FH caused a significant ($p < 0.001$) reduction in LDH leakage when compared to cells exposed to 10 μg/ml DEP only (Figure 3B). In contrast, our data show a non-significant change in LDH leakage in keratinocytes exposed

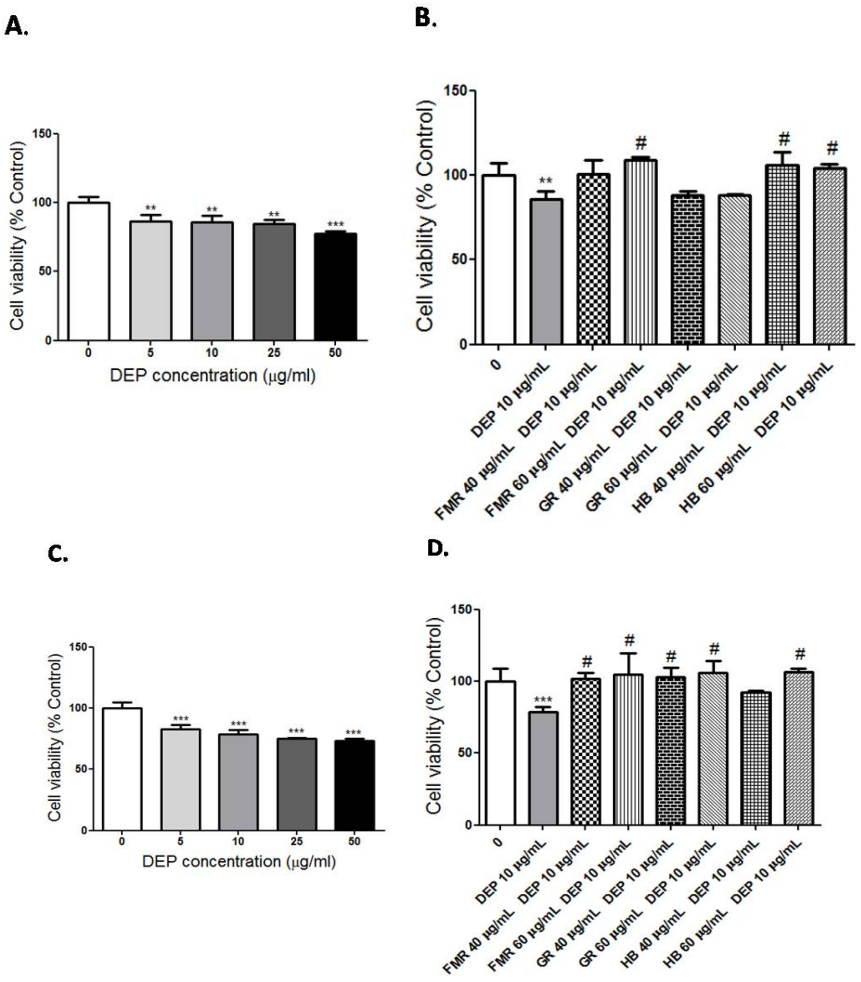

Figure 2: Effect of aqueous extracts of rooibos and honeybush on DEP-induced cytotoxicity. The viability of fibroblasts (A, B) and keratinocytes (C, D) were determined as described in the materials and methods. Values are mean ± SEM of three different experiments done in triplicate ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as significant difference as compared to the controls. #$p < 0.01$ as significant difference as compared to 10 µg/ml DEP.

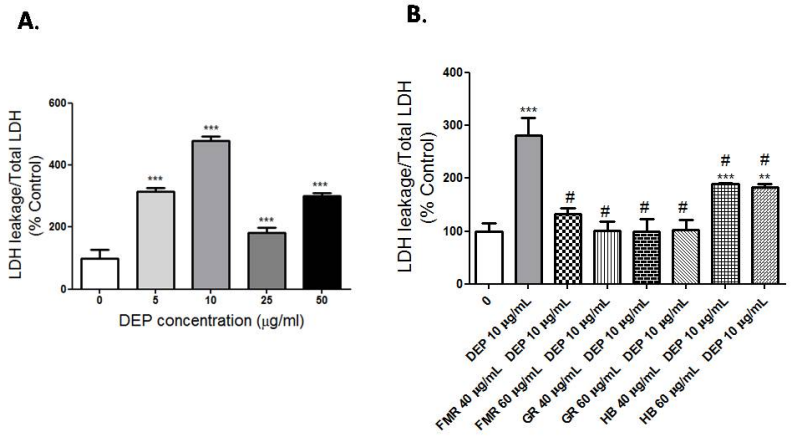

Figure 3: Effect of aqueous extracts of rooibos and honeybush on DEP-induced membrane damage. Lactate dehydrogenase (LDH) levels were determined in (A, B) fibroblasts and (C, D) keratinocytes, Values are mean±SEM of three different experiments done in triplicate ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as significant difference as compared to the controls. #$p < 0.01$ as significant difference as compared to 10 µg/ml DEP.

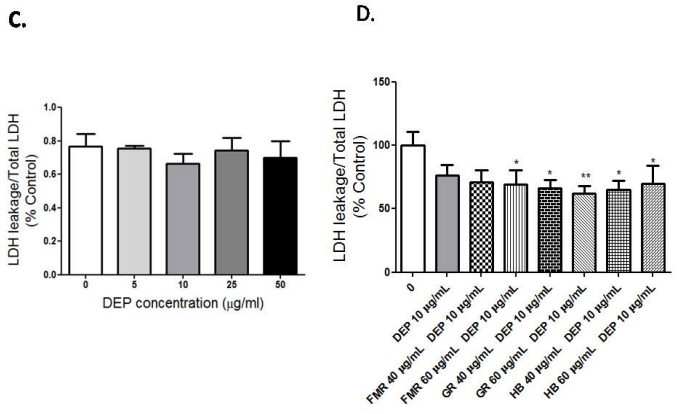

Figure 3 (Cont.)

to 5-50 μg/ml DEP after 4 h exposure compared to control (Figure 3C). Also, the pre-treatment with 40 and 60 μg/ml FR, GR or FH did not have any significant effect on LDH leakage compared to keratinocytes exposed to 10 μg/ml DEP only (Figure 3D).

3.4. Rooibos tea and Honeybush extracts attenuate DEP-induced pro-inflmmatory effect

This study shows that exposure to 10 μg/ml DEP caused significant ($p < 0.001$) increase in TNF αmRNA expression in fibroblasts when compared to control (Table 3). Pre-treatment of fibroblasts with 40 and 60 μg/ml FR, GR and FH resulted in significant ($p < 0.001$) reduction in TNFα mRNA expression when compared to 10 μg/ml DEP only (Table 3). No significant change in ATF4 mRNA expression in fibroblasts exposed to 10 μg/ml DEP when compared to control (Table 3). Also, pre-treatment with rooibos and honeybush extracts do not offer any significant effects on ATF4 mRNA expression in fibroblast when compared to cells exposed to 10 μg/ml DEP (Table 3).

Table 3: Effects of DEP on pro-inflammatory cytokines expression in fibroblasts and keratinocytes pre-treated with the herbal tea extracts

Treatment	Inflammatory markers ($\Delta CT = CT_{target} - CT_{\beta\text{-actin}}$)			
	Fibroblasts		Keratinocytes	
	TNFα	ATF4	IL-6	IL-8
Negative Control	16.10 ± 0.22	3.82 ± 0.15	14.08 ± 0.43	9.96 ± 0.14
DEP (10 μg/ml)	14.44 ± 0.19***	3.66 ± 0.45	13.47 ± 0.07*	9.41 ± 0.10**
Fermented Rooibos (FR):	15.99 ± 0.27$^{\#}$	3.61 ± 0.04	14.49 ± 0.16$^{\#}$	10.09 ± 0.23$^{\#}$
40 μg/ml + DEP	15.97 ± 0.17$^{\#}$	3.38 ± 0.11*	14.07 ± 0.17$^{\#}$	10.76 ± 0.14$^{\#}$
60 μg/ml +DEP				
Green Rooibos (GR):	16.38 ± 0.13$^{\#}$	3.91 ± 0.13	14.30 ± 0.19$^{\#}$	10.48 ± 0.21$^{\#}$
40 μg/ml + DEP	15.46 ± 0..50$^{\#}$	3.67 ± 0.09	14.30 ± 0.14$^{\#}$	10.31 ± 0.06$^{\#}$
60 μg/ml + DEP				
Fermented Honeybush (FH):	15.25 ± 0.20$^{\#}$	3.60 ± 0.07	14.04 ± 0.15$^{\#}$	12.18 ± 0.15$^{***\#}$
40 μg/ml + DEP	15.76 ± 0.33$^{\#}$	3.80 ± 0.09	15.17 ± 0.22$^{\#}$	9.98 ± 0.08$^{\#}$
60 μg/ml + DEP				

Note: Values in columns are mean ± SD of three ($n = 3$) different experiments done in triplicate. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ indicate significant differences when compared to the control. $^{\#}p < 0.01$ indicates significant differences when compared to 10 μg/ml DEP. DEP – Diesel Exhaust Particles; and ΔCT – Cycle Threshold. .

In keratinocytes, exposure to 10 μg/ml DEP caused significant ($p < 0.01$) increase in IL-6 and IL-8 mRNA expressions when compared to control (Table 3). Pre-treatment with 40 and 60 μg/ml FR, GH and FH caused significant ($p < 0.001$) decrease in IL-6 and IL-8 mRNA expressions in keratinocytes when compared to cells exposed to 10 μg/ml DEP only (Table 3).

3.5. DEP exposure enhanced Nrf2 nuclear migration in response to oxidative stress

We investigated the activation and nuclear migration of Nrf2 after DEP exposure. Our data indicate increased nuclear localization of Nrf2 in fibroblasts (Figure 4A) and keratinocytes (Figure 4B) after DEP exposure.

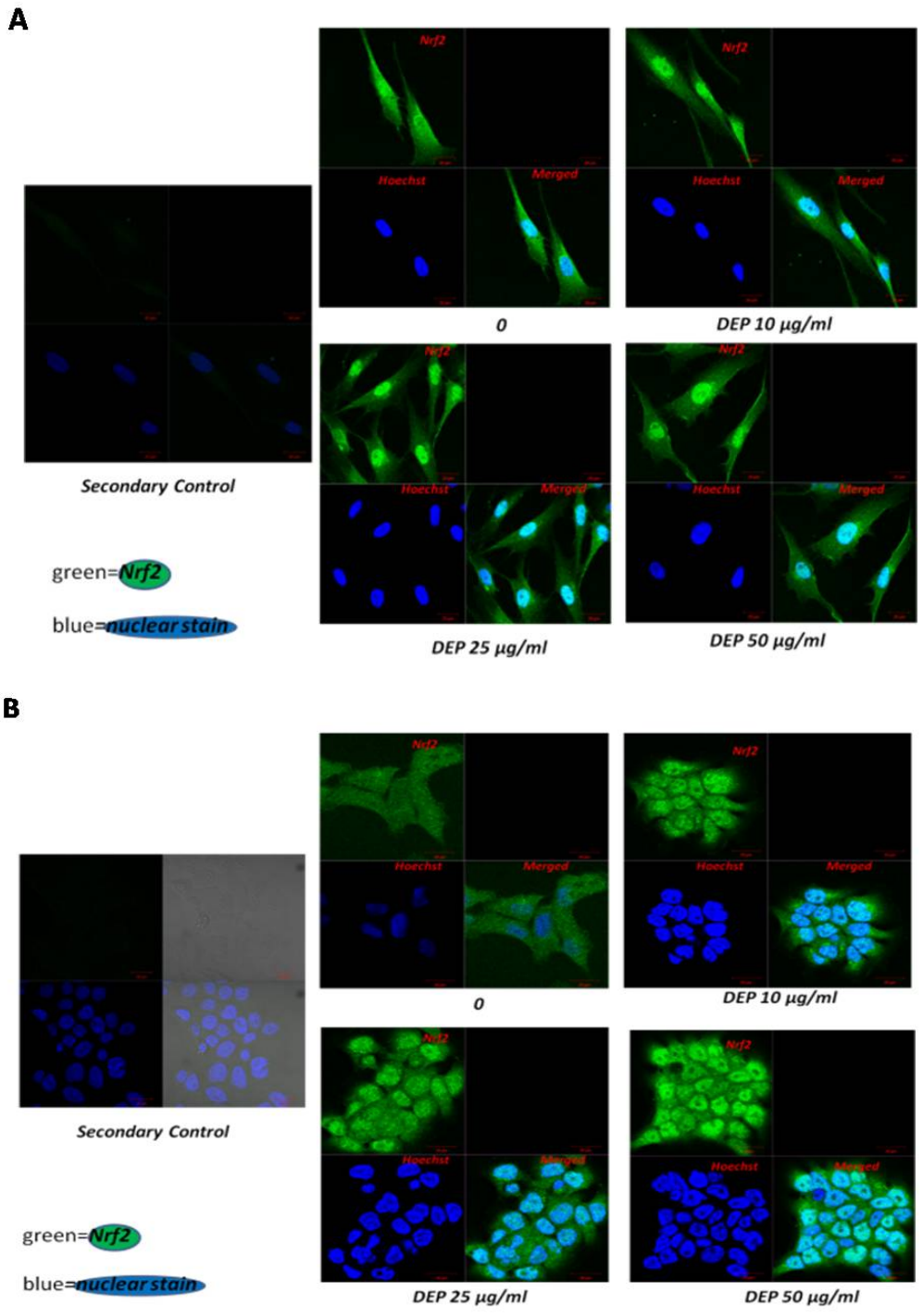

Figure 4: Effect of DEP on Nrf2 nuclear translocation. Nrf2 migration after DEP exposure was assessed in (A) Fibroblasts and (B) keratinocytes. The image represents a total of three experiments ($n = 3$)

3.6. DEP induction of phase I and phase II enzymes is altered by Rooibos tea and Honeybush extracts

We found that DEP induced significant ($p < 0.05$) increase in NQO1 expression at 10 μg/ml in fibroblasts (Figure 5A) and keratinocytes (Figure 5B) when compared to the control. Except at 40 μg/ml FMR, pre-treating fibroblasts with FR, GR or FH before 10 μg/ml DEP exposure did not produced any significant change in NQO1 mRNA (Figure 5A). However, pre-treatment of keratinocytes with the extracts caused significant ($p < 0.01$) reduction in NQO1 gene expression compared with 10 μg/ml DEP only (Figure 5B).

Exposure of fibroblasts (Figure 5C) and keratinocytes (Figure 5D) to 10 μg/ml DEP caused significance ($p < 0.01$) increase in CYP1B1 mRNA level when compared to control. Pre-treatment with FR, GR and FH mitigate against DEP-induced CYP1B1 mRNA expression in both cell lines (Figure 5C and D).

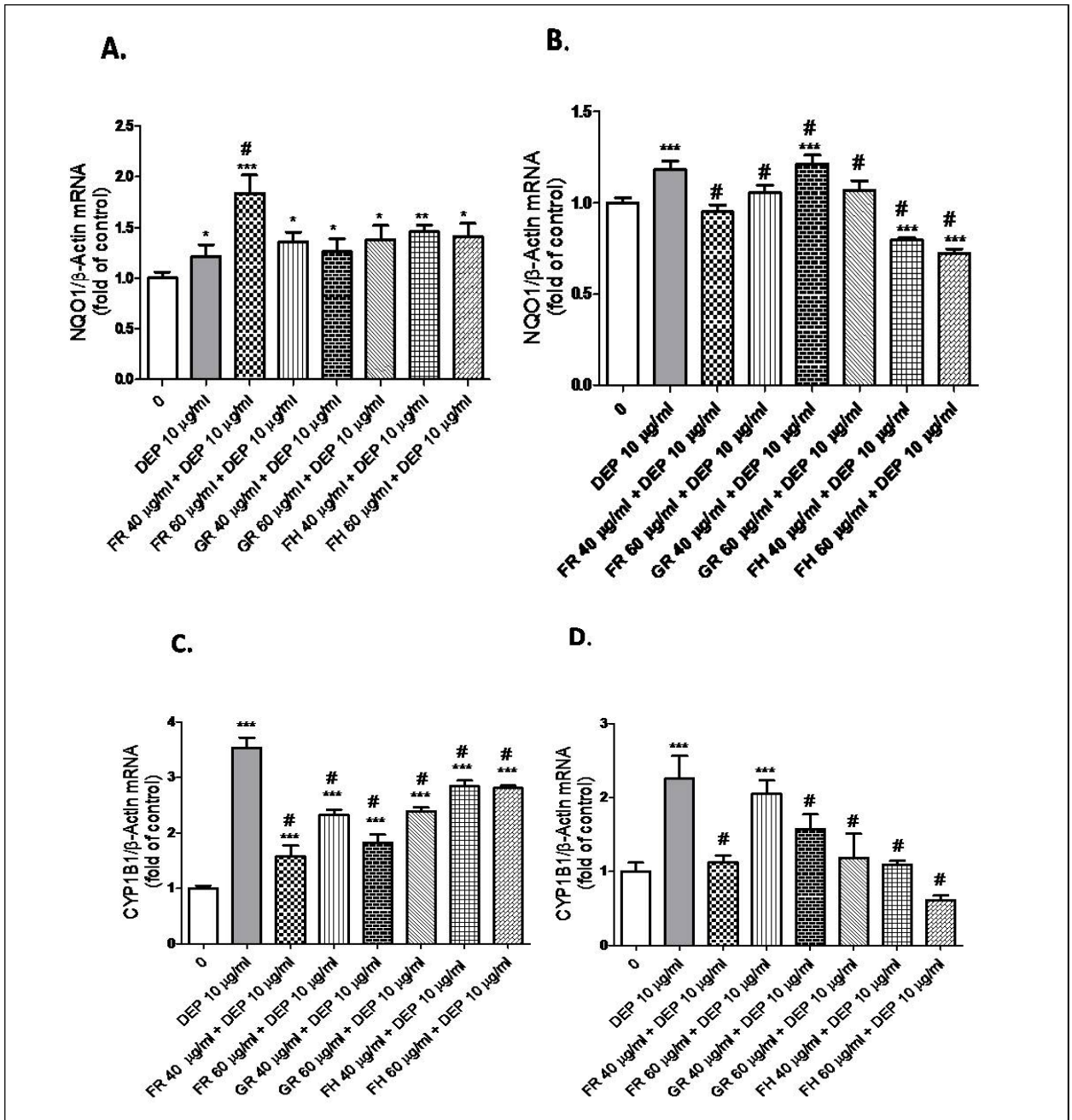

Figure 5: Effect of Rooibos and Honeybush extracts on DEP-induced NQO1 and CYPIBI mRNA levels. NQOI mRNA in (A) fibroblasts and (B) keratinocytes and CYP1B1 mRNA levels in (C) fibroblasts and (D) keratinocytes were determined by RT-qPCR. Values are mean ± SEM of three different experiments done in triplicate ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ significantly different when compared to the controls, and $^{\#}p < 0.001$ significantly different when compared to 10 μg/ml DEP.

3.7. Rooibos tea and Honeybush extracts activate Wnt/β-catenin signaling in respond to DEP-induced damage

The results showed that 10 μg/ml DEP caused 1.15- and 1.39- fold decrease in phospho-β-catenin and GSK-3β protein expression, while it induced Wnt 3 protein expression by 1.72- fold in fibroblasts when compared to control (Figure 6A). Pretreatment of fibroblasts with either 40 or 60 μg/ml FR, GR and FH caused significant reduction in phospho-β-catenin and GSK-3β protein expression when compared to 10 μg/ml DEP (Figure 6A). In contrast, the extracts pre-treatment caused significant increased in Wnt 3 protein expression in fibroblasts when compared to 10 μg/ml DEP (Figure 6A).

We also found that 10 μg/ml DEP caused 3.59- and 1.36- fold increase in keratinocytes phospho-β-catenin and GSK-3β protein expressions, respectively when compared to control (Figure 6B). Pretreatment with either 40 or 60 μg/ml FR, GR or FH, however, caused significant reduction in both phospho-β-catenin and GSK-3β protein expressions in the keratinocytes compared to 10 μg/ml DEP (Figure 6B). In contrast, the extracts pretreatment caused significant increase in Wnt 3 protein expression when compared to 10 μg/ml DEP (Figure 6B).

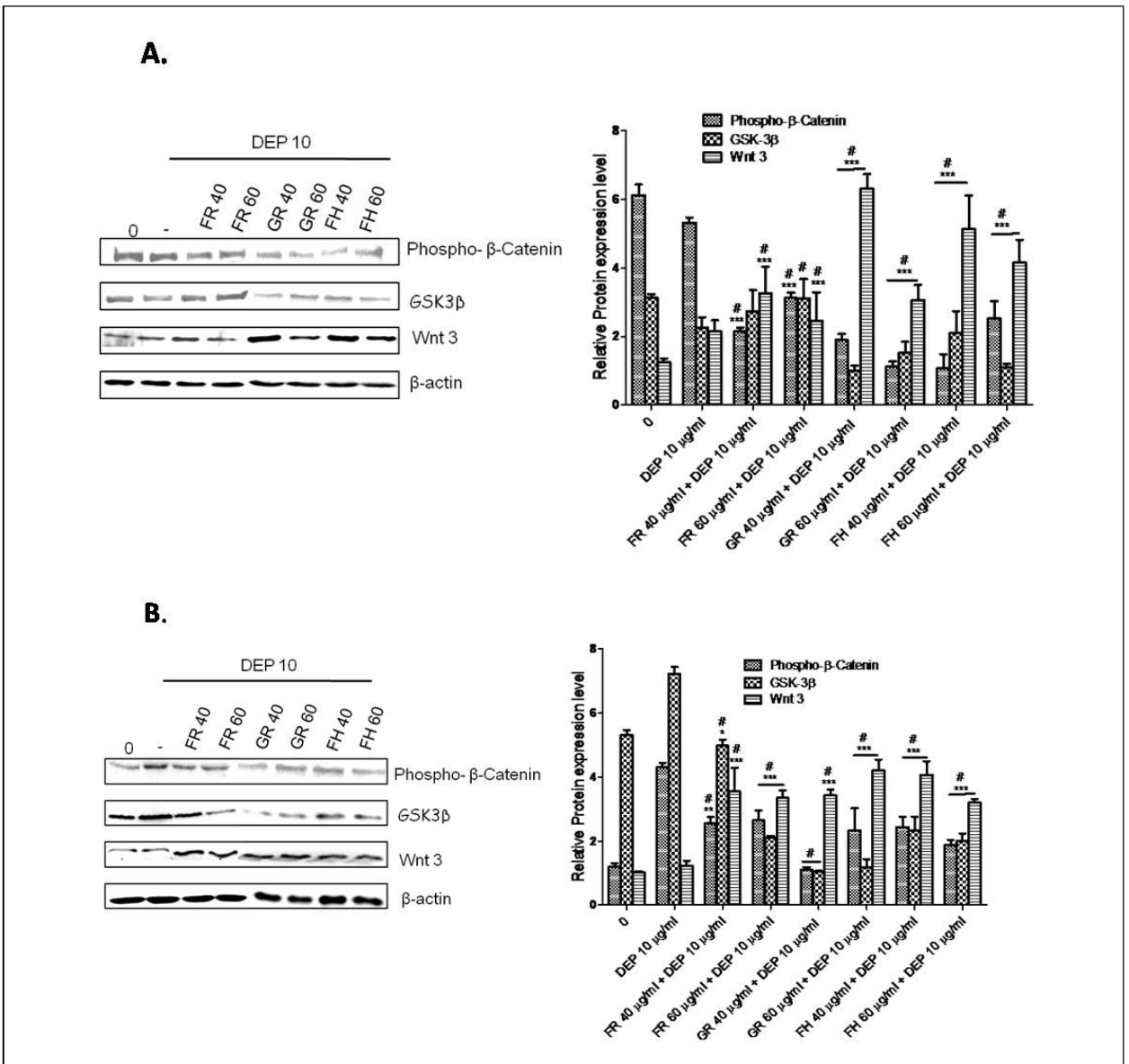

Figure 6: Effect of Rooibos and Honeybush extracts on Wnt/β-catenin signaling in the presence of DEP. Wnt/β-catenin pathway was assessed in (A) fibroblasts and (B) keratinocytes by western blot. Values are mean ± SEM of three different experiments done in triplicate ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ significantly different when compared to the controls, and #$p < 0.001$ significantly different when compared to 10 μg/ml DEP

4. Discussion

Our data indicate that DEP SRM 2975 from an industrial forklift engine induce significant cytotoxic, pro-oxidative and pro-inflammatory effects in both human skin fibroblasts and keratinocytes. The data also show that aqueous extracts of two Rooibos preparations; fermented and green rooibos, and Honeybush teas protect these cells from the pro-oxidant and pro-inflammatory effects of DEP (SRM 2975).The cytotoxicity, pro-oxidative and pro-inflammatory effects of DEP have been reported in several studies (Oluyede *et al*., 2021; Lawal *et al*., 2015; Frikke-Schmidt *et al*., 2011; and Tseng *et al*., 2015). Different studies have shown DEP to induce ROS production, leading to oxidative stress with resultant damage to macromolecules and decrease GSH/GSSG ratio (Forchhammer *et al*., 2012; and Lawal *et al*., 2019a**)**. Our results were consistent with these former findings as we found a significant increase in ROS production in a time-dependent manner in both cell lines. We also found increased in protein carbonyl formation, conjugated diene levels, lipid peroxidation and reduced GSH/GSSG ratio in both cell lines in response to DEP exposure, probably as a result of increased ROS production. One consequence of increased ROS production and macromolecular oxidative damage is increased cytotoxicity as evidence in decrease cell survival and increase cell membrane damage. We found decreased in the population of the surviving cells in both fibroblasts and keratinocyte as well as increase LDH leakage in fibroblasts in response to DEP exposure. These results were in agreement with previous studies, using different cell lines, where exposure to DEP has been shown to induce cell death and LDH leakage (Lawal *et al*., 2015; Lawal *et al*., 2019a; and Tseng *et al*., 2015).

The reduced glutathione (GSH) is an important reducing agent in the cells and plays an important role in maintaining the redox homeostasis of the cell. The depletion in the GSH level as a result of increase oxidative stress leads to GSSG accumulation and a drop in the GSH/GSSG ratio. The disturbed redox equilibrium could activate several redox-sensitive signaling pathways such as the MAP kinases and the NF-κB cascade to activate cytokine and adhesion molecule expression. Studies have shown that DEP induce JNK, P38MAPK and NF-κB activities which lead to cytokine and chemokine production in vivo and in vitro (Lawal *et al*., 2019b; and Kim *et al*., 2016). Indeed, DEP have been shown to induce the expression of IL-6, IL-8, IL-1á and TNF α mRNA in systemic sclerosis skin keratinocytes and IL-1β mRNA in normal dermal keratinocytes (Mastrofrancesco *et al*., 2014). In addition, other in vitro studies have reported induced expression of IL-6, IL-8, MCP-1, ICAM-1 and VCAM-1 in cells exposed to DEP (Lawal *et al*., 2015; and Frikke-Schmidt *et al*., 2011). In agreement with these previous studies, our data show that DEP increase TNFα and ATF4 mRNA expression in fibroblasts, and also increase IL-6 and IL-8 mRNA expression in keratinocytes probably in response to the disturbed redox homeostasis as a result of elevated ROS production.

In order to counteract the effects of oxidative stress, cells are equipped with both enzymic and non-enzymic antioxidants that neutralized the excess ROS. Antioxidants use different mechanisms such as free radical scavenging, inhibition of free radical generating enzymes, metal ion chelating and prevention of lipid peroxidation to maintain redox homeostasis (Pradedova *et al*., 2011). The triggering of this antioxidant defense is regulated by the transcription factor p45-NFE2 related transcription factor 2 (Nrf2) through the modulation of its proteasomal degradation in the cytosol and translocation into the nucleus (He *et al*., 2020) Several studies have shown the induction of Nrf2 and Nrf2-regulated cytoprotective enzymes in response to DEP exposure (Lawal *et al*., 2015; and Tseng *et al*., 2015) . Nrf2 activation has been reported to mediate the activation of the aryl hydrocarbon receptor (AhR) pathway, which regulate the cytochrome P450 gene (CYP) expression (Haarmann-Stemmann *et al*., 2012). Palkova *et al* (2015) reported that PAHs and polar aromatic compounds also contribute to DEP associated AhR-mediated activity. A study has shown that PAHs increase CYP1B1 expression in primary keratinocyte and primary dermal fibroblast (Borland *et al* 2014). Jaguin *et al* (2015) also reported an induction in CYP1B1 expression in macrophage after exposure to DEP SRM 1975 extract. Consistent with these earlier studies, we found that exposure to DEP triggers a nuclear translocation of Nrf2, and the induction of NQO1 and CYP1B1 mRNA expression in both fibroblasts and keratinocytes, probably in response to oxidative stress and polycyclic aromatic hydrocarbons (PAHs)-generated electrophilic activation of AhR.

The effective use of exogenous antioxidant against DEP-induced cytotoxicity has been widely reported in many studies (Frikke-Schmidt *et al*., 2011; and Yin *et al*., 2013). Recently, we have shown that rooibos and honeybush extracts protect against DEP-induced cytotoxicity in HMEC (Lawal *et al*., 2019a). However, no work as yet reported on the use of antioxidant phytochemicals from plants such as rooibos and honeybush teas against DEP-induced cytotoxic effects in fibroblasts and keratinocytes.

Here we hypothesize that aqueous extracts from fermented and green rooibos, and honeybush teas will protect against DEP-induced pro-oxidative and pro-inflammatory effects in human skin fibroblasts and keratinocytes. Our data indicate that pre-treatment with FR, GR or FH extracts caused a reduction in ROS generated by DEP with the consequent attenuation of oxidative damage to macromolecules in both fibroblasts and keratinocytes in support of our hypothesis. In contrast, pre-treatment with the extracts caused elevation in the GSH/GSSG ratio, thereby implicating GSH elevation as a possible mechanism involve in the protective action of the extracts in both cell lines. These findings were in line with previous clinical and animal studies, which have reported a reduction in plasma levels of thiobarbituric acid reactive substance (TBARS), conjugated dienes and elevation in the GSH/GSSG ratio following consumption of rooibos herbal tea (Marnewick *et al.*, 2011; and Lawal *et al.*, 2019b). The extracts pre-treatment also enhanced cell survival and protect against membrane damage, especially in fibroblasts, highlighting the importance of different sensitivity of each cell line to DEP toxicity. Furthermore, in support of our hypothesis, we found that the FR, GR and FH extracts pre-treatment was able to attenuate the expression of pro-inflammatory genes, such as a TNFa mRNA expression in fibroblasts and IL-6 and IL-8 mRNA expression, in keratinocytes which may likely be due to the antioxidative effect of the extracts. This was consistent with the previous findings that have reported on the anti-inflammatory effects of rooibos and honeybush teas and/or their constituents polyphenols in both in vitro and in vivo models (Lawal *et al.*, 2019a and b). Interestingly, we found that the extracts pre-treatment in fibroblasts, except at 40 μg/ml FR, did not alter NQO1 expression in the presence of DEP, but caused a reduction in NQO1 expression in keratinocytes. Similarly, extracts pre-treatment caused a reduction in CYP1B1 expression in fibroblasts and in keratinocytes.

The Wnt signaling pathway is activated in the presence of appropriate ligand to stimulate the desphosphorylation of β-catenin with its consequent migration into the nucleus to increase transcription of β-catenin-dependent genes (Vikram *et al.*, 2014**).** The activation of Wnt/β-catenin signaling has been reported to enhance extracellular matrix remodelling, wound healing and tissue repair (Whyte *et al.*, 2012). The extracts activate the Wnt/β-catenin signaling by enhancing the dephosphorylation of β-catenin through the reduced expression of GSK-3β, a protein that induced β-catenin phosphorylation and its ubiquitylation for degradation. The activation of the Wnt/β-catenin signaling by the extracts may be responsible in part for their cytoprotective effect in response to the cytotoxicity of DEP.

5. Conclusion

In summary, this study demonstrates that the presence of aqueous extracts of FR, GR and FH offer protection against DEP harmful effects probably by a mechanism that might involve the activation of the Wnt/β-catenin signaling. Future work is required to elucidate further how the extracts protect against DEP-induced effects in both fibroblasts and keratinocytes. Rooibos and honeybush teas intake could serve as a health promoting beverages to prevent skin damage and disease caused as a result of DEP exposure.

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