

Potential Therapeutic Benefits of Green and Fermented Rooibos (*Aspalathus linearis*) in Dermal Wound Healing

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ABSTRACT

The process of wound healing constitutes an ordered sequence of events that provides numerous opportunities for therapeutic intervention to improve wound repair. Rooibos, *Aspalathus linearis*, is a popular ingredient in skin care products, however, little scientific data exists exploring its therapeutic potential. In the present study, we evaluated the effects of fermented and aspalathin-enriched green rooibos in various *in vitro* models representative of dermal wound healing. Treatment of RAW 264.7 macrophages with fermented rooibos resulted in increased nitric oxide production as well as increased levels of cellular inducible nitric oxide synthase and cyclooxygenase-2, which are typical markers for classically activated macrophages. In contrast, the green extract was devoid of such activity. Using glycated gelatin as a model to mimic diabetic wounds, only the green extract showed potential to reduce cyclooxygenase-2 levels. Considering the role of reactive oxygen species in wound healing, the effects of rooibos on oxidative stress and cell death in human dermal fibroblasts was evaluated. Both fermented and green rooibos decreased cellular reactive oxygen species and attenuated apoptotic/necrotic cell death. Our findings highlight several properties that support the therapeutic potential of rooibos, and demonstrate that green and fermented rooibos present distinctly different properties with regards to their application in wound healing. The proinflammatory nature of fermented rooibos may have therapeutic value for wounds characterised with a delayed initial inflammatory phase, such as early diabetic wounds. The green extract is more suited to wounds burdened with excessive inflammation as it attenuated cyclooxygenase-2 levels and effectively protected fibroblasts against oxidative stress.

Introduction

Disruption to the integrity of the skin initiates a multistep process that ultimately leads to reconstruction of the damaged area and reestablishment of the barrier function of the skin. From a histological perspective, the wound healing process is defined as three distinctive yet partly overlapping phases, namely, inflammation, proliferation, and tissue remodelling [1]. Disruption at any stage prevents the sequential process and subsequently derails the healing process, giving rise to chronic wounds.

Macrophages are recognised to play a critical part in the repair of skin by clearing damaged tissue constituents, killing pathogens, and producing a variety of growth factors that induce collagen deposition, angiogenesis, and wound closure. Recruitment and activation of macrophages is thus important for proper wound healing, however, persistence of activated macrophages leads to hyper-inflammation and excessive oxidative stress that results in the delayed resolution of the inflammatory phase and subsequent impairment of diabetic wound healing [2].

Aspalathus linearis (Burm.f.) R.Dahlgren (Leguminosae), commonly referred to as rooibos, is a South African herbal tea that has become increasingly popular worldwide. The plant is endemic to a restricted region of South Africa where it is extensively cultivated for both domestic and international markets. Rooibos is considered unique due to the presence of aspalathin and aspalalinin, two monomeric flavonoids exclusive to rooibos [3]. In addition, rooibos also contains nothofagin, a rare 3-dehydroxydihydrochalcone glucoside.

Once harvested, rooibos twigs are bruised and allowed to ferment for a few hours, resulting in a change from green to a characteristic red colour and development of the distinctive flavour and aroma typical of rooibos tea. This processing, however, significantly reduces the polyphenol content, including that of aspalathin, which is extensively oxidised to dihydroiso-orientin, and, subsequently, decreases the antioxidant activity [4]. Attempts to maximise the aspalathin and antioxidant content of rooibos for medicinal and cosmetic applications has led to development of a patented extraction procedure from unfermented rooibos (also known as green rooibos) and a final product with greater aspalathin content and improved antioxidant activity than that of the traditional fermented extract [5].

Although there are numerous reports mentioning the use of rooibos in the treatment of skin ailments including wounds, no direct scientific evidence exists demonstrating the efficacy and safety of rooibos as a therapeutic strategy in the management of chronic wounds such as diabetic and pressure wounds. Since inflammation and oxidative stress are implicated in the pathogenesis of chronic wounds, it may be argued that the several publications attributing such activities to rooibos provides some support for potential wound healing activity, however, the majority of these *in vivo* studies evaluate the pharmacological properties from the perspective of an herbal tea and not as a dermally applied preparation [6, 7].

In the present study, we have investigated the effect of rooibos on selected aspects relevant to chronic wounds using various *in vitro* models, including some representative of the chronic/diabetic wound environment. While it is acknowledged that such simple cell-based models may not incorporate the complex cellular interactions present in the *in vivo* situation, these *in vitro* assays provide a suitable platform to identify putative therapeutic mechanisms in preparation for more context driven *in vivo* studies. In addition, we also compare the therapeutic potential of the aspalathin-enriched green rooibos extract to that of the traditional fermented extract.

Our findings indicate significant differences in the properties of fermented and green rooibos, which appear to extend beyond the aspalathin/antioxidant activity, and highlight the need to evaluate the therapeutic potential of rooibos in a context-dependent manner, as the wound healing process comprises multiple aspects, many of which reciprocate to opposing extremes during the course of the healing process and subsequently redefine the therapeutic advantage of any given treatment.

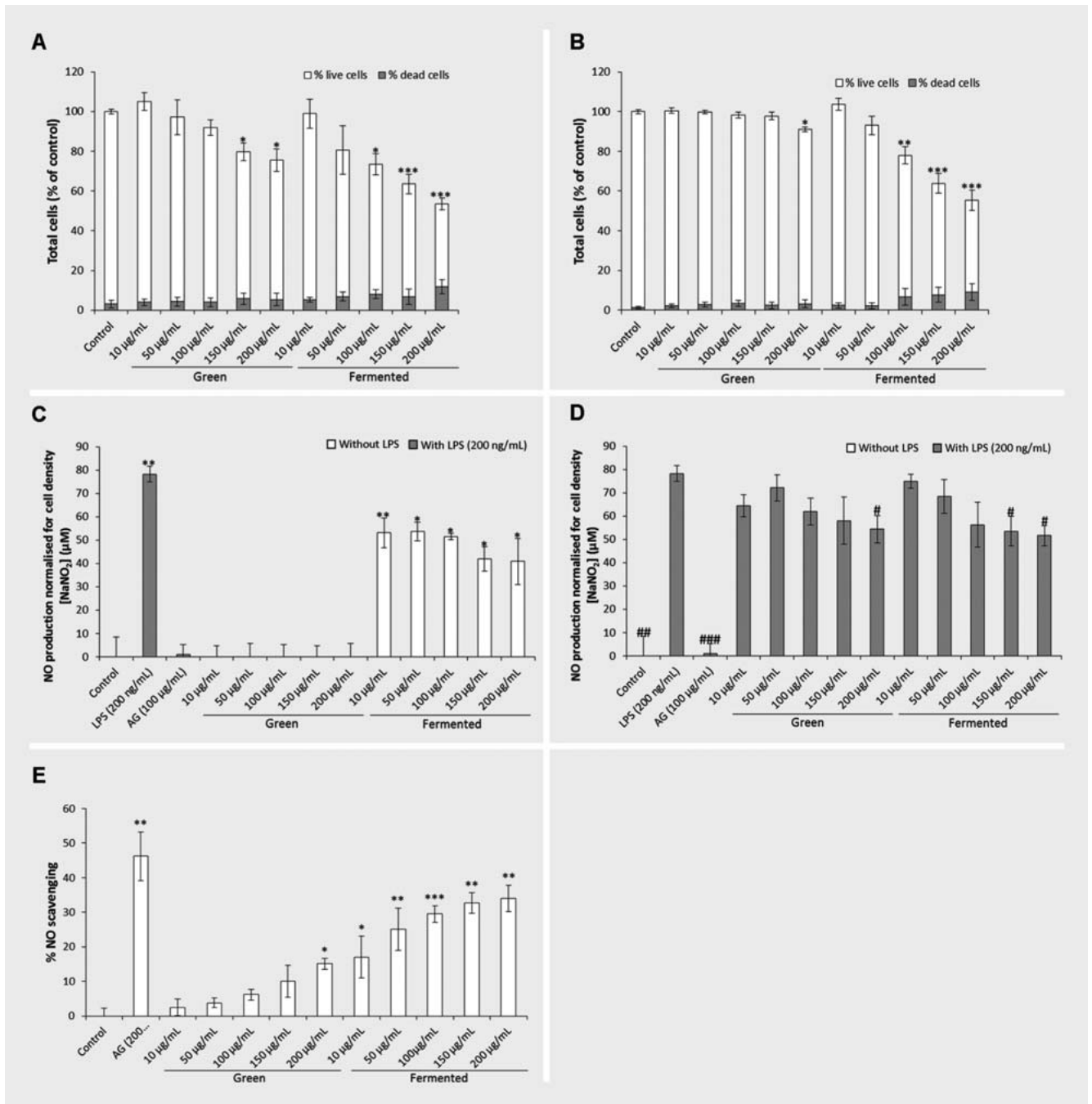
Results and Discussion

Both green and fermented rooibos extracts dose-dependently decreased macrophage cell density, however, the levels of propidium iodide (PI) positive cells were, in general, not significantly altered, suggesting that inhibition of proliferation rather than cytotoxicity was the primary contributing factor (► Fig. 1A). Considering the principle function of wound macrophages to produce nitric oxide (NO), a toxic gas known to inhibit cell proliferation, we assessed the production of NO as a potential antiproliferatory mechanism. As illustrated in ► Fig. 1C, fermented rooibos extract significantly increased nitrate levels in the culture medium, while the green extract was completely devoid of such activity. Although the magnitude of the positive response was significantly less than that of lipopolysaccharide (LPS), the lowest fermented rooibos concentration illustrated in ► Fig. 1C (10 µg/mL) already produced a near maximal effect. Further dilution revealed a measurable dose-dependent increase in NO production in the range of 0.1 to 10 µg/mL with an EC₅₀ value of approximately 500 ng/mL (data not shown). Since both extracts inhibited macrophage proliferation, the observed difference between the green and fermented extracts indicates that the antiproliferatory mechanism is either independent of NO or that the two extracts inhibit proliferation via distinct mechanisms.

To further explore the role of macrophage activation and NO in the antiproliferatory activity of rooibos, macrophages were simultaneously treated with the extracts and LPS, a potent inducer of inducible nitric oxide synthase (iNOS) and concomitant NO production. There was no meaningful difference in the antiproliferatory effect of fermented rooibos when co-treated with LPS (► Fig. 1B). In contrast, LPS treatment almost completely attenuated the antiproliferatory activity of green rooibos extract.

We also established the effect of rooibos extracts on LPS-induced NO production, indicating only a marginal decline in NO production when cells were treated with LPS (► Fig. 1D), an effect which may be attributed to the NO scavenging activity of the extracts (► Fig. 1E). These results show that the signalling cascade of LPS is not significantly altered in the presence of the rooibos extracts and further confirm that the macrophage anti-mitogenic activity of green rooibos is not a result of NO production.

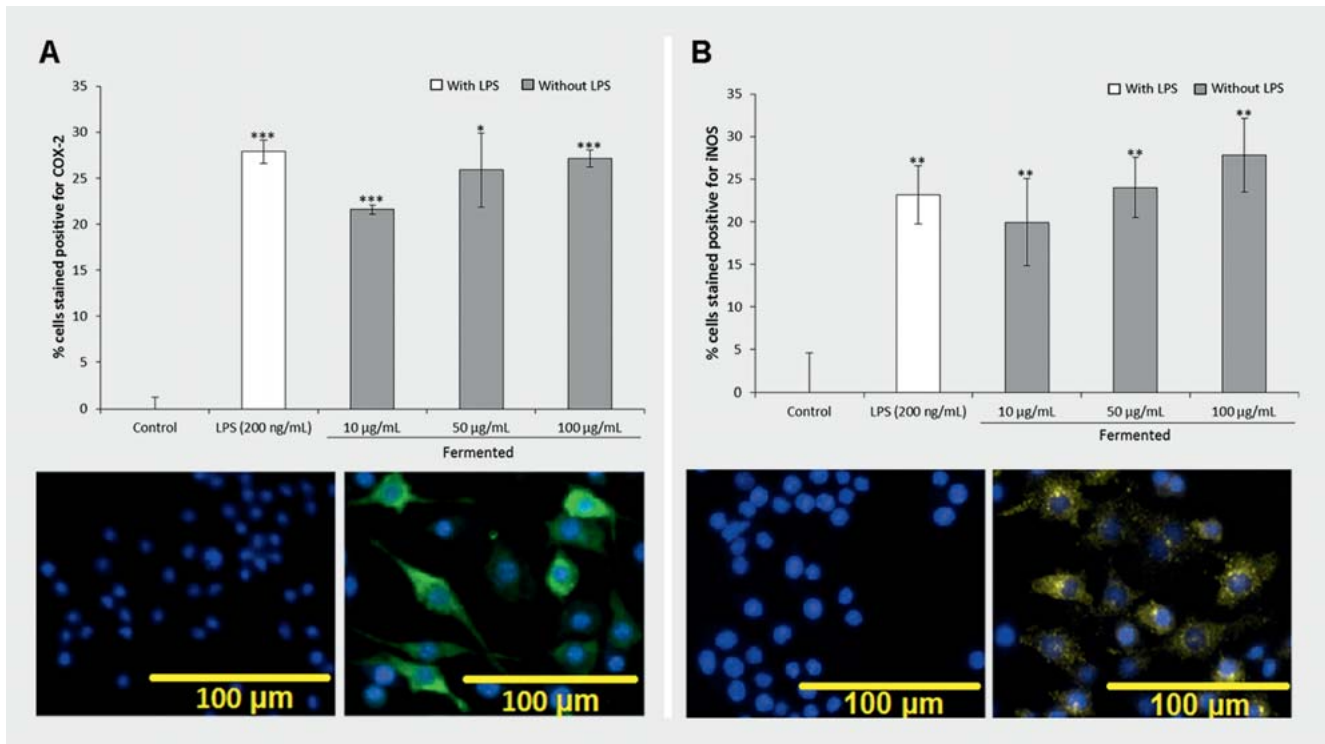
Wound macrophages are characterised by a dynamic plasticity passing from classical to alternative activated states as the wound matures. To confirm that fermented rooibos induces a proinflammatory phenotype, as suggested by the increased NO production, we evaluated the effects of fermented rooibos on the induction of markers typically associated with classically activated macrophages. Both iNOS and cyclooxygenase-2 (COX-2) protein levels were significantly increased in RAW macrophages treated with fermented rooibos (► Fig. 2), thus corroborating the proinflammatory nature of this extract. In support of our findings, a study by Hendricks and Pool [8] similarly reported that *ex vivo* human white blood cells treated with a fermented rooibos extract increased interleukin 6 (IL-6) and interferon gamma (IFN-γ) production. Both IL-6 and IFN-γ may be considered biomarkers for classically activated macrophages. In contrast to the fermented extract, green rooibos did not reveal any significant increase in iNOS and COX-2 levels (data not shown).



► **Fig. 1** A comparison of RAW 264.7 cytotoxicity and NO (nitric oxide) production (A–D) and NO scavenging activity (E) of *A. linearis* green and fermented extracts. Cytotoxicity was measured using Hoechst 33342 and PI staining (A) without LPS (lipopolysaccharide) and (B) with LPS. NO production was measured using the Griess reaction (C) without LPS and (D) with LPS. LPS and amino-guanidine (AG) were used as positive controls for the stimulation and inhibition of NO production, respectively, and all values were normalised for cell density using the crystal violet assay (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ compared to the control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.005$ compared to LPS).

Considering that prolonged inflammation is a primary characteristic of chronic wounds, one may intuitively anticipate that treatment with fermented rooibos holds a risk to exacerbate delayed healing by supporting a hyper-inflammatory state. However, several studies document that treatment of wounds with agents that improve monocyte recruitment and classical activation significantly improve healing outcomes. The macrophage ac-

tivating agent glucan, a beta-1,3-linked glucose polymer that interacts with polysaccharide receptors and subsequently triggers cell activation, has been shown to improve several healing parameters [9,10]. The external application of low concentrations of bacterial endotoxins, natural ligands for macrophage activation via Toll-like receptors (TLRs), is also reported to have potential wound healing properties [11].



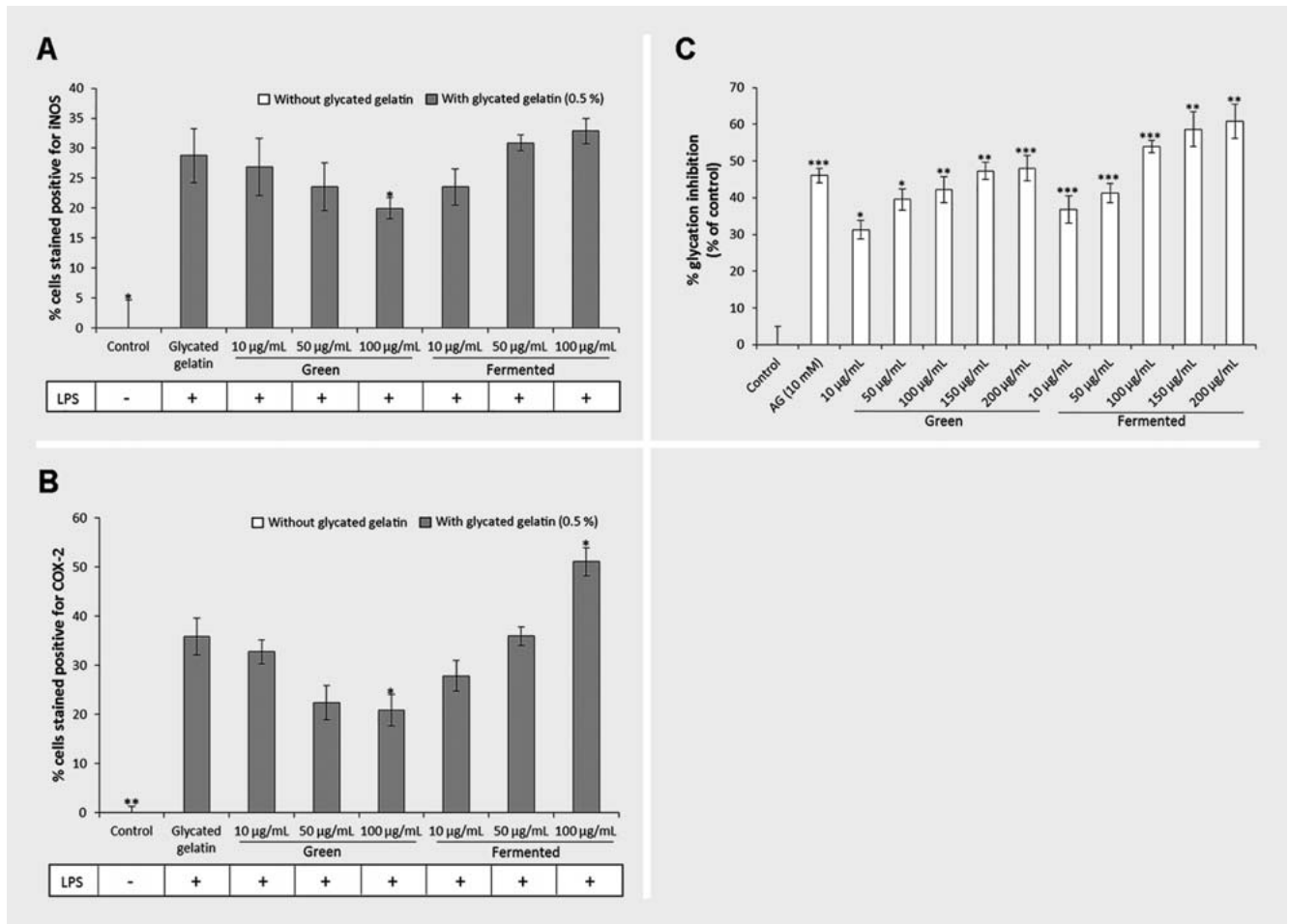
► **Fig. 2** Immunofluorescence using RAW 264.7 cells comparing (A) COX-2 antibody staining showing the percentage of cells stained positive for COX-2 and fluorescent micrographs captured using the 20× objective for the control (left) and 100 µg/mL fermented hot water extract (right) where Hoechst 33342 stained nuclei are shown in blue and COX-2 staining of the cytoplasm in green and (B) iNOS antibody staining showing the percentage of cells stained positive for iNOS and fluorescent micrographs captured using the 20× objective for the control (left) and 100 µg/mL fermented hot water extract (right) where Hoechst 33342 stained nuclei are shown in blue and iNOS staining in the cytoplasm in yellow. LPS (lipopolysaccharide) was included as a positive control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ compared to the control).

In further support of the therapeutic potential of proinflammatory macrophages, it has been shown that exogenous administration of classically activated (M1) macrophages to normal and diabetic wounds can promote the healing response [12, 13]. Similarly, rapid recruitment and activation of macrophages into murine wounds are reported to accelerate tissue repair [13]. Moreover, depletion of macrophages during early wound healing negatively impacts wound closure [14], illustrating that the initial proinflammatory macrophage function is critically involved in the healing process and also contributes to the later phases of wound healing. Together these findings argue in favour that some degree of proinflammatory activation is essential to promote proper wound healing and supports the hypothesis that any pathological condition that dampens the initial inflammatory response may lead to an insufficient reaction to stimulate the progression to the next phase of wound healing [15]. Furthermore, therapeutic interventions that “jump-start” such weakened responses could hold a clinical benefit. In this context, external application of fermented rooibos may be considered to have the potential to enhance wound healing, however, it will clearly depend on the nature of the wound.

To more directly explore the therapeutic potential of rooibos in the treatment of diabetic wounds, we treated macrophages with a combination of LPS and glycated gelatin to simulate two characteristic features of such wounds, namely, microbial infection and

protein glycation, respectively. Characterisation of this model revealed many features typical of diabetic wounds including, amongst others, increased levels of iNOS and COX-2 (► **Fig. 3A, B**). Rooibos treatment under these conditions revealed two opposing trends. For the green extract, a decrease in the levels iNOS and COX-2 was evident, although only the highest tested concentration reached statistical significance ($p < 0.05$). In contrast, and perhaps not entirely unexpected given its proinflammatory nature, the fermented extract produced a dose-dependent increase in these parameters, however, only COX-2 levels at the highest tested concentration was statistically significant ($p < 0.05$) (► **Fig. 3A, B**). Evaluating the effects of rooibos on the process of protein glycation revealed that both extracts could significantly inhibit the formation of advanced glycation end products (AGEs) (► **Fig. 3C**). Given the negative association of AGEs on wound healing, inhibition of protein glycation may have therapeutic potential, especially as a long-term treatment.

Hyper-inflammation in chronic wounds no doubt contributes to oxidative stress, which can trigger cell death. Apoptosis of bystander cells, such as dermal fibroblasts, negatively impact early wound healing, as these cells provide essential components necessary for optimal healing. Excessive fibroblast apoptosis has been proposed as a mechanism for the impairment of diabetic wounds [16]. Rooibos is well known for its strong antioxidant capacity, with aspalathin being one of the many phenolic compounds con-



► **Fig. 3** Immunofluorescent detection of (A) iNOS and (B) COX-2 antibody staining in RAW 264.7 cells following treatment with glycated gelatin and *A. linearis* and (C) glycation inhibitory potential of the extracts. LPS (lipopolysaccharide; 200 ng/mL) was included in the treatments for iNOS and COX-2 staining only [$*p < 0.05$, $**p < 0.01$, $***p < 0.005$ compared to glycated gelatin (A, B) or compared to the control (C)].

tributing to this activity. Rooibos may thus be anticipated to improve the survival of wound fibroblasts challenged with excessive ROS. To evaluate the protective potential of rooibos in this setting, MRHF human foreskin fibroblasts were treated with *tert*-butyl hydroperoxide (TBHP), and the effects of fermented and green rooibos on oxidative stress and cell death were evaluated using the fluorescent dye CellROX orange and Hoechst/Annexin V-FITC/PI staining, respectively. A dose-dependent inhibition in ROS is evident for both the fermented and green extracts, with green rooibos having slightly improved antioxidant activity relative to the fermented extract (► **Fig. 4**), an effect that appears somewhat insignificant relative to the considerably higher phenolic content, especially aspalathin, of the green extract (Table 1S, Supporting Information).

To mimic the external exposure of wound fibroblasts to the oxidative environment produced by external sources such as activated macrophages, we evaluated the effects of rooibos on MRHF dermal fibroblasts in direct combination with TBHP. Both green and fermented extracts improved cell density relative to the TBHP only treatment, with the green extract having a slightly better effect (► **Fig. 5A**). Cell death, as assessed using PI and Annexin V

staining, indicated a robust decline in the percentage of cells staining positive for these markers, confirming the protective activity of rooibos (► **Fig. 5B, C**). A similar trend was observed when fibroblasts were pretreated with the rooibos extracts and omitted when adding TBHP (► **Fig. 5D, E**), suggesting that the protective effects may involve some degree of cellular adaptation, resulting in a greater resistance against oxidative stress. These results support a protective capacity for rooibos against oxidative stress-induced cell death, a property that can enhance wound healing [17].

Taken together, our findings highlight several features regarding the therapeutic potential of rooibos for wound healing. Green and fermented rooibos present distinctly different properties with regards to macrophage function. The proinflammatory activity of the fermented extract is completely devoid in the green extract. Similarly, the antiproliferatory mechanism of these extracts appear different and both activities show no apparent correlation to the phenolic content of the extracts (including aspalathin). Given that macrophage function is critical to proper wound repair and a recognised therapeutic target, rooibos may be considered to possess potential wound healing activity and warrants further studies. It is important to emphasise that the process of wound

healing is highly context driven (disease status of the patient, degree and type of infection, and so on) and that the precise molecular mechanisms leading to diminished wound repair remain to be fully elucidated, hence, it is necessary to evaluate the therapeutic potential of any treatment within a particular context.

Materials and Methods

Reagents, chemicals, cell culture, and maintenance

All reagents and chemicals were from Sigma unless otherwise stated. RAW 264.7 murine macrophage-like cells and human dermal fibroblasts (MRHFs) were from American Type Culture Collection and Cellnex, respectively. Cells were cultured in a humidified (5% CO₂) incubator at 37 °C in DMEM:10% FBS with 1% (v/v) penicillin-streptomycin in 10-cm culture dishes. Cells were passaged when at 70% confluence. All experiments were performed in TPP 96-well culture plates. DMEM and FBS were from HyClone Laboratories. Penicillin-streptomycin and PBS containing Ca²⁺ and Mg²⁺ from Lonza.

Plant extract preparation

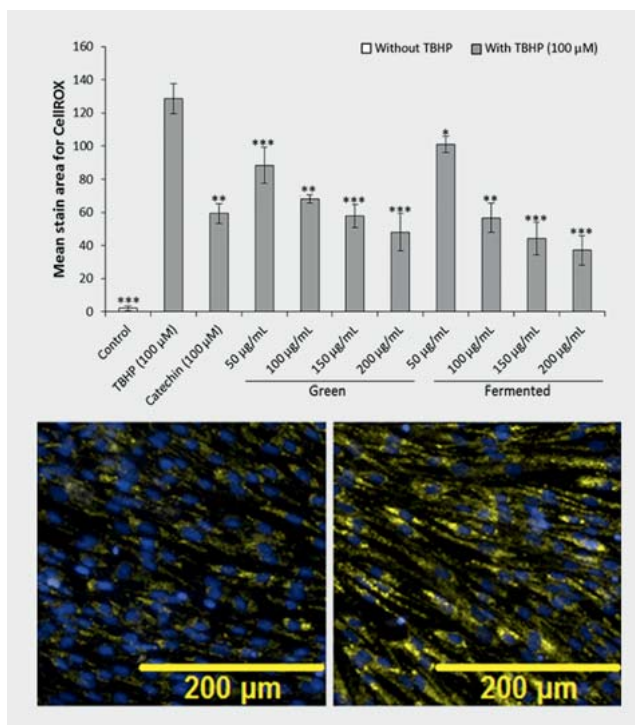
A. linearis extracts were a kind donation from Profs. E. Joubert and D. de Beer from the Agricultural Research Council, Stellenbosch, South Africa. Green and fermented rooibos herbal tea were procured from Rooibos Ltd. (Clanwilliam, South Africa). The plant material was extracted with hot water as described by [18]. Briefly, this entailed the addition of 1 L boiling water to 100 g plant material and incubation in a water bath for 30 min at 93 °C, followed by filtration through Whatman No. 4 filter paper. The extracts were freeze-dried. Retention samples of the green and fermented rooibos extracts were coded ALI_L0091_1028B1_1604 and ALI_L0193_1029B1_1604, respectively. Major phenolic compounds were quantified as described by [19]. Stocks were prepared fresh weekly in DMSO (40 mg/mL).

Cytotoxicity screening

RAW 264.7 macrophages (2 × 10⁴ cells/well in 96-well plates) were seeded overnight and treated for 48 h. The percentage of live and dead cells was determined using Hoechst 33342 (purity ≥ 98% by HPLC and TLC) and PI (purity ≥ 94% by HPLC) dual staining, respectively, as described by [20] with slight modifications. Medium was replaced with 50 μL Hoechst 33342 (5 μg/mL in PBS), incubated for 10 min at room temperature (RT) and protected from light. Immediately before image acquisition, 50 μL of PI (100 μg/mL in PBS) were added.

Nitric oxide production

RAW 264.7 macrophages were seeded (1 × 10⁵ cells/well in 96-well plates) overnight and treated for 24 h at 37 °C in the presence or absence of 200 ng/mL LPS (from *Escherichia coli*), as described by [21] with slight modifications. Amino-guanidine (AG) at 100 μg/mL (purity ≥ 98%) was included as a positive control for NO inhibition. The NO produced was quantified by adding 50 μL Griess reagent to 50 μL of spent culture medium and incubating at RT for 10 min. Absorbance was measured at 540 nm using a BioTek PowerWave XS spectrophotometer (BioTek-Instruments).



► **Fig. 4** Mean stain area of CellROX in MRHF fibroblasts treated with both TBHP (*tert*-butyl hydroperoxide) and *A. linearis*. Fluorescent micrographs were captured using a 10× objective comparing the control (left) with TBHP-treated cells (right). Catechin (100 μM) was included as a positive control. P values indicating significances are *p < 0.05, **p < 0.01, ***p < 0.005 compared to TBHP.

Nitric oxide scavenging assay

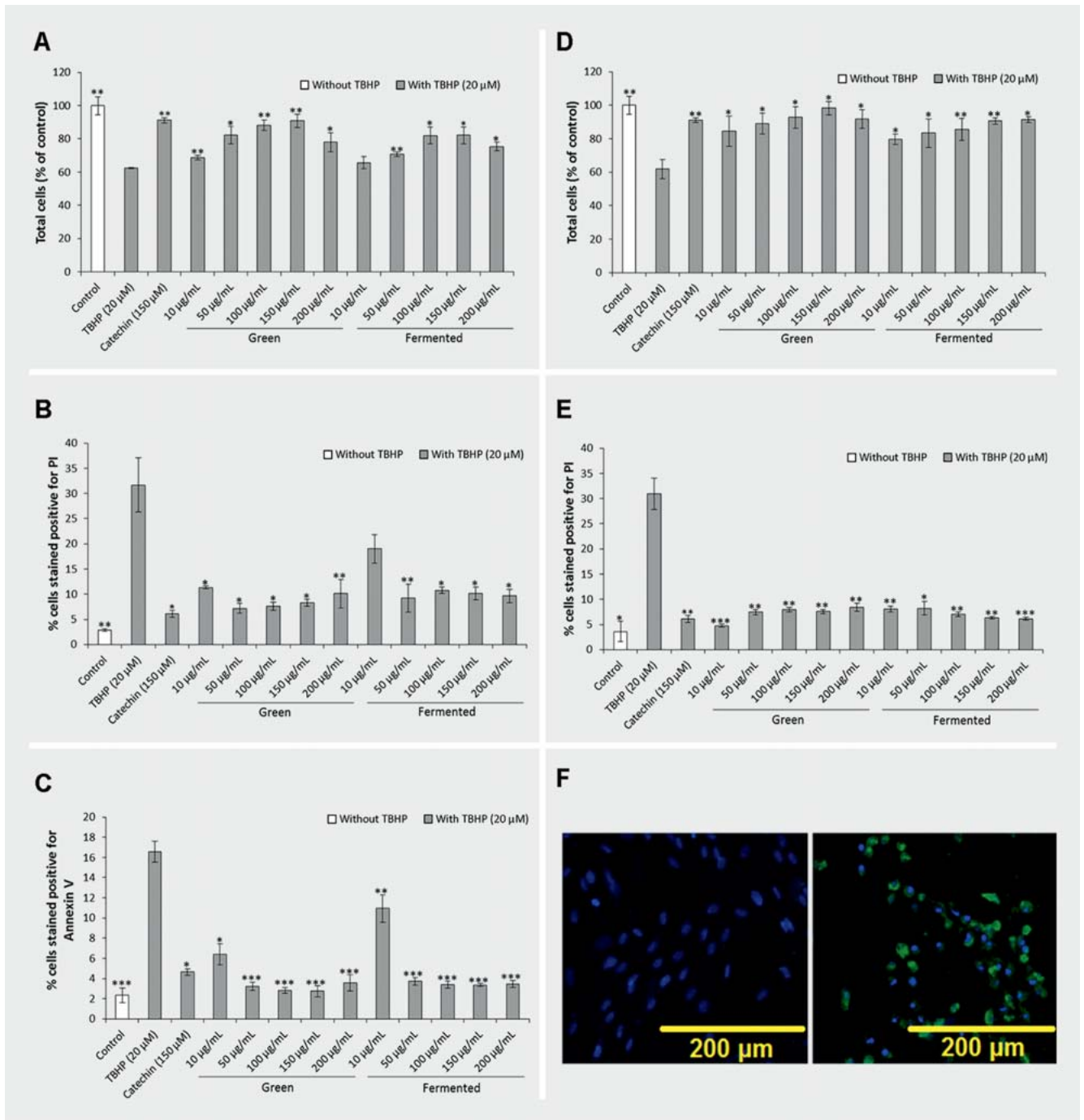
Using a modification of the method described previously by [22], rooibos extract (50 μL/well) was incubated with 50 μL of sodium nitroprusside (10 mM, purity ≥ 99%) at RT under direct light for 150 min followed by the addition of 100 μL Griess reagent, as described previously with modifications [22]. Absorbance was measured after 10 min for the NO production assay. AG (200 μg/mL) was included as a positive control and the percentage of NO scavenged was calculated as follows:

$$\% \text{ NO scavenging} = \frac{A - B}{A} \times 100$$

where A and B are the absorbance values of the reference and test sample, respectively.

Immunofluorescent detection of cyclooxygenase-2 and inducible nitric oxide synthase

RAW 264.7 macrophages were seeded (3 × 10⁴ cells/well in 96-well plates) overnight and exposed to treatments for 24 h at 37 °C. Glycated gelatin [prepared by autoclaving 20% (w/v) tissue culture grade endotoxin-free gelatin from porcine skin with 1 M glucose for 60 min] was included in the medium as a surrogate protein for glycated collagen to mimic the diabetic state (model previously characterised by our lab). Cells were fixed and stained



► Fig. 5 A comparison of the effects of *A. linearis* on oxidative stress-induced apoptosis in MRHF fibroblasts comparing (A) total cell number with (B) cell death and (C) apoptosis (detected using Annexin V-FITC staining) for cells pretreated with *A. linearis* for 24 h, followed by treatment with *A. linearis* in the presence of TBHP (*tert*-butyl hydroperoxide) for a further 24 h. The same experiment was repeated; however, cells were pretreated with *A. linearis* for 24 h after which *A. linearis* was removed from the culture medium and cells were treated with only TBHP for a further 24 h comparing (D) total cell number with (E) cell death (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ compared to TBHP). F Fluorescent micrographs captured using a 10 \times objective comparing the control (left) with TBHP-treated cells (right) where Hoechst 33342 stained nuclei are shown in blue and apoptotic cells are stained green with Annexin V-FITC.

according to the manufacturer's instructions, with modifications. For COX-2 antibody staining, cells were fixed by adding formaldehyde directly to the culture medium and permeabilised with cold methanol (100 μ l) at -20°C for 10 min. For iNOS staining, cells

were fixed and permeabilised with the IntraPrep permeabilization reagent kit (Beckman Coulter). Cells were washed twice with 1% BSA/PBS and blocked at RT using a solution containing 3% BSA/PBS and 0.2% Triton-X100 for 45 min. All antibodies were from

Cell Signalling Technology and prepared in 1% BSA/PBS. For COX-2 and iNOS, cells were incubated for 60 min at 37 °C in 50 µL Cox2 (D5H5) XP Rabbit mAb (Alexa Fluor 488 Conjugate) (1:800) and iNOS (D6B6S) rabbit mAb (PE Conjugate) (1:800), respectively. After washing 3× with PBS, nuclei were stained with Hoechst 33342 as described previously. Fluorescent micrographs were captured as described below.

Glycation inhibition

The glycation inhibitory potential of *A. linearis* was assessed using BSA as described by [23]. The fluorescence of glycated BSA was measured using a Fluoroskan Ascent FL fluorometer from Thermo LabSystems at 370 nm_{Ex}/440 nm_{Em}.

Oxidative stress using CellROX

The ability of *A. linearis* to protect MRHF cells against oxidative stress was assessed using TBHP and CellROX Orange (Thermo Fisher Scientific). MRHF cells were seeded (8×10^3 cells/well) overnight and pretreated as indicated for 24 h at 37 °C. Oxidative stress was then induced by adding TBHP (100 µM) to the medium for 2 h at 37 °C as described previously by [24], with modifications. Cells were stained with CellROX Orange (5 µM) as per the manufacturer's instructions together with Hoechst 33342 (5 µg/mL in PBS) for 30 min at 37 °C, protected from light, and fluorescent micrographs were captured.

Apoptosis inhibition

MRHF human skin fibroblasts were seeded (8×10^3 cells/well in 96-well plates) overnight and pretreated with *A. linearis* for 24 h at 37 °C using only 5% FBS in culture medium. TBHP (20 µM) was then added to induce apoptosis and the cells were incubated for a further 24 h. The percentage of live, dead, and apoptotic cells were measured with Hoechst 33342, PI, and the Annexin V-FITC kit from Miltenyi Biotec, respectively, as described by [20], with modifications.

Imaging and data and statistical analysis

Fluorescent micrographs were acquired using Molecular Devices ImageXpress Micro XLS and analysed using the Multi-Wavelength Cell Scoring module from MetaXpress 6.1 Software. The following filter sets were used: DAPI (Hoechst 33342), Texas Red (PI), TRITC (PE, CellROX Orange), and FITC (Alexa Fluor 488, Annexin V-FITC). Nine image sites per well were acquired for all protocols. The 10× objective was used for cytotoxicity, CellRox, and apoptosis; 20× for COX-2 and iNOS.

Results are reported as the mean ± standard deviation, and experiments were conducted three times, each in triplicate. Using Microsoft Excel, the ANOVA and Student's t-test were implemented to test for statistically significant differences ($p < 0.05$) between the means of the data sets [25].

Supporting information

The quantities of major phenolic compounds in the green and fermented extracts as well as the corresponding HPLC chromatograms are available as Supporting Information.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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