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Attenuation of oxidative stress and artificial wound closure in C2C12 myoblasts induced by sequential extracts of *Boerhavia diffusa*

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Keywords
Boerhavia diffusa; cytotoxicity; migration; oxidation; wounds

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Abstract

Objectives Whole plants of *Boerhavia diffusa* L. are widely used medicine in Ghana and other tropical countries, for the treatment of wounds and other ailments. The aim of the study was to determine the ability of sequential extracts of *B. diffusa* to influence oxidation and wound closure in myoblast cells *in vitro*.

Methods Sequential extracts were prepared from the whole plant using four solvents of increasing polarity (hexane, ethyl acetate, methanol and water). Cytotoxicity was determined using the sulforhodamine B staining assay, phase-contrast microscopy, plasDIC microscopy and live–dead staining. Extracts were tested for their ability to reduce 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidation and mediate cell migration after artificial wound generation in C2C12 myoblast cells using the scratch wound assay.

Key findings All extracts indicated negligible cytotoxicity (IC_{50} > 100 μg/ml), and microscopic evaluation showed no difference from negative controls. AAPH induced a 2.87-fold increase in reactive oxygen species compared to the negative control. Pretreatment with 100 μg/ml of the extracts reduced AAPH-induced oxidation to 1.70-fold of the untreated controls ($P < 0.001$). Wound closures in the methanol and water extract treatments were 18.08% and 20.76% higher than the negative control, respectively ($P < 0.01$).

Conclusions These findings indicate that the hexane, methanol and water extracts of *B. diffusa* whole plant promote artificial wound healing and protection against oxidation *in vitro* and therefore warrant further research into its mechanisms of wound healing.

Introduction

The restoration of injured tissue is essential for the survival of all species. Most tissues, such as muscle, have an intrinsic ability to regenerate after injury, but the healing process is slow and often incompletely resolved.[1] A series of complex and overlapping events characterize the process of wound healing. Muscle damage, either through trauma or innate genetic defects, triggers an acute inflammatory response that is characterized by rapid neutrophil and macrophage infiltration, elevated secretion of inflammatory cytokines and increased production of reactive oxygen species (ROS).[1,2] These events are quickly followed by phagocytosis of damaged cells, activation, differentiation and migration of satellite cells to the injury site, and terminal differentiation of myoblasts into myotubes.[2]

Two factors which could affect the healing of damaged or wounded muscle tissue are cellular migration and ROS release. Cellular migration plays pivotal roles in virtually every aspect of human survival, including inflammation, cancer and injury.[3,4] Myoblast migration is particularly essential in myogenesis and regeneration, allowing for myoblast alignment and their fusion into myotubes. This process is necessary for complete restoration of health and function to injured muscle tissue.[2,5] Myoblastic cell lines, such as C2C12, are widely used to study the effects of chemical substances on skeletal muscle growth and differentiation *in vitro*. Furthermore, these cells are particularly

suitable for studying myoblast migration, an essential component of muscle regeneration after injury.[6,7]

Reactive oxygen species released during the process of inflammation plays regulatory roles in vital processes, serving as secondary messengers by influencing the cellular redox status.[8] Under physiological conditions, ROS release is regulated by intracellular antioxidants such as superoxide dismutase and catalase.[8,9] However, there are instances where the antioxidant defence system of the body is overwhelmed leading to excessive ROS production and induction of oxidative stress. The latter results in cellular damage and impairment of wound or tissue healing.[9–11] Substances which promote cellular migration or inhibit ROS-related activities could be successful in enhancing healing of injured tissue.

Boerhavia diffusa L., a member of the Nyctaginaceae family, is a perennial herb. It is used as a medicinal plant in areas such as the tropical parts of Africa, South America and India. Almost all parts of the herb, the leaves, stem and roots, are used in treating wounds, as well as conditions of the reproductive system, jaundice, kidney problems, skin problems, eye diseases and inflammation,[12] as well as neurological conditions such as epilepsy.[13] Researchers have demonstrated 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, protection against DNA damage, α-amylase inhibitory effect of the plant[14,15] and anti-inflammatory properties (nitric oxide and tumour necrosis factor-α inhibition).[16] Although this plant has also been shown to have a host of beneficial biological effects, including anti-angiogenic and antidiabetic activity as well as protection against development of cardiac hypertrophy,[17–19] its effect on cell migration has not been investigated yet.

Phytochemical constituents, including alkaloids, steroids, phenols, glycosides, reducing sugars, amino acids and flavonoids, have been detected in methanol whole plant extracts.[20] Subsequent studies have identified various phenolic acids and flavones such as quercetin, kaempferol, as well as boeravinone E, G and H.[21,22]

The aim of the study was to evaluate the effect of four sequential extracts of *B. diffusa* whole plants for potential oxidative protection and cellular migratory activity in C2C12 myoblasts in order to determine its suitability as a remedy for the management of wounds.

Materials and Methods

Chemicals and reagents

All reagents, unless specified otherwise, were obtained from Sigma–Aldrich, USA. Hexane, ethyl acetate and methanol were purchased from Merck (Pty) Ltd, South Africa.

Source of plant material

Whole plants of *Boerhavia diffusa* L. (Nyctaginaceae) were collected from the University of Cape Coast, Ghana, and its neighbouring communities and authenticated by Mr. Francis Otoo from the University of Cape Coast School of Biological Sciences herbarium, where a voucher specimen (UCCH0041215) is deposited. The samples were washed thoroughly, air-dried at room temperature, finely powdered using a grinder (Glen Creston, UK) and stored in sterile airtight containers. Ethical approval was obtained from the University of Pretoria's Faculty of Health Sciences Research Ethics Committee to carry out the study (194/2017).

Preparation of extracts

Extraction was performed sequentially with four different solvents in increasing polarity as described by Arokiyaraj *et al.*[23] Powdered plant material (10 g) was sonicated in 100 ml hexane, shaken for 30 min on an electronic shaker and incubated at 4 °C for 24 h. The solvent was decanted and the marc air-dried. The marc was re-extracted with ethyl acetate, methanol and water following the same procedure, although no further sonication took place. Filtrates were dried *in vacuo* with a rotary evaporator (Buchi Rotavapor R-200) at 40 °C, with exception of the water extract which was freeze-dried (Labconco 31 Freezone 6). Gravimetric yields were determined, and extracts stored at −20 °C until needed.

Phytochemical screening and analysis

Thin-layer chromatography analysis was performed according to the methods described by Stahl.[24] Ultraperformance liquid chromatography and time-of-flight mass spectrometry (UPLC-TOF-MS) were conducted on a Waters instrument coupled in tandem to a Waters SYNAPT G1 HDMS mass spectrometer and used to generate accurate mass data. The chromatographic separation followed the procedure described by Parkar *et al.*[25]

Table 1 Phytochemical composition of *B. diffusa* extracts

Chemical component	Plant extract			
	H	E	M	W
Alkaloids	+	+	–	+
Saponin	–	+	+	–
Coumarins	–	–	–	–
Flavonoids	+	+	+	–
Phenols	+	+	+	–
Glycosides	+	+	+	–
Tannins	–	+	–	–
Terpenes	+	–	+	–

H, hexane; E, ethyl acetate; M, methanol; W, water. +, Present, –, Absent.

MassLynx 4.1 (SCN 872) software was used to control the hyphenated system as well as for data manipulation.

Cell culture and maintenance

C2C12 myoblasts were purchased from the American Tissue Culture Collection (ATCC, CRL-1772) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum (FCS), 100 μg/ml streptomycin and 100 mg/l penicillin at 37 °C in a humidified incubator containing 5% CO_2. Confluent cells were rinsed with phosphate-buffered saline (PBS) and enzymatically detached with trypsin/Versene solution for 5 min. Cells were harvested through centrifugation (200 *g*, 5 min) and resuspended in 10% FCS-supplemented DMEM for counting using trypan blue exclusion.

Evaluation of cytotoxicity of extracts

Sulforhodamine B staining

The assay was performed as described by Vichai and Kirtikara with minor modifications to volumes used.[26] Cells, seeded at a density of 1×10^4 cells/well in 96-well plates in

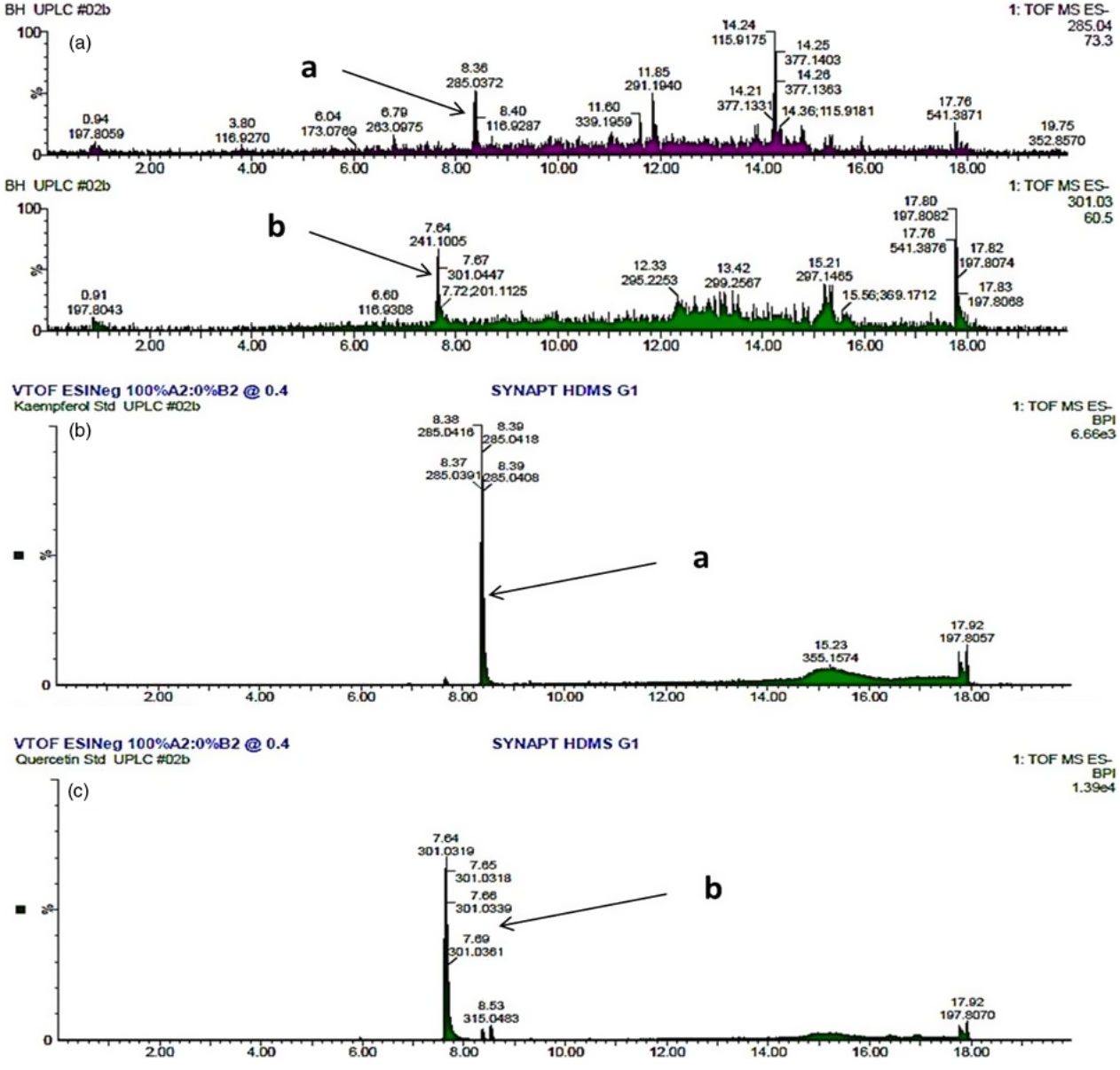


Figure 1 The extracted mass chromatograms of the hexane extract of *Boerhavia diffusa* (A) depicting the presence of kaempferol (a) and quercetin (b); the base peak intensity chromatograms of reference standards kaempferol (B) and quercetin (C). All data are presented in ESI-negative mode.

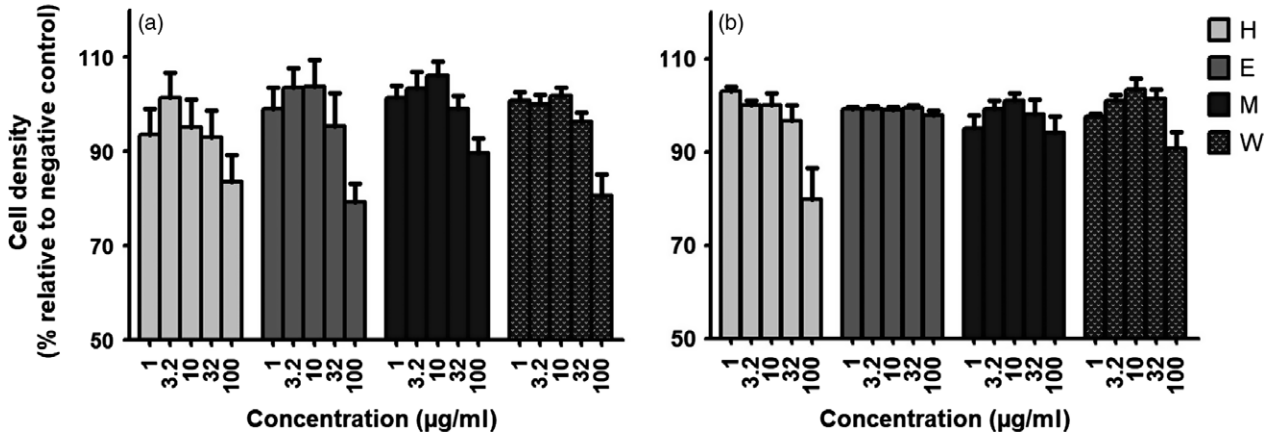


Figure 2 Cell density of C2C12 myoblast cells treated with the hexane (H), ethyl acetate (E), methanol (M) and water (W) extracts of *B. diffusa* after (a) 24-h and (b) 48-h exposure.

10% FCS-supplemented medium, were incubated overnight to allow for attachment. Cells were exposed to 1, 3.2, 10, 32 and 100 μg/ml of each extract in reaction (prepared in FCS-free DMEM, 200 μl reaction mixture) for 24 and 48 h. Saponin (1% in reaction), DMSO (0.5% in reaction) and DMEM were used as positive, vehicle and negative controls, respectively. A blank consisting of 5% DMEM was used to account for sterility and background noise. Treated cells were fixed with cold 10% (w/v) trichloroacetic acid solution in reaction overnight. Fixed cells were washed three times with running tap water and stained with 0.057% sulforhodamine B (in 1% acetic acid solution) for 30 min. Excess sulforhodamine B was removed by washing with 1% acetic acid, and plates were dried in a low-temperature oven for 60 min. The protein-bound dye was solubilized in 10 mM Tris-base solution by shaking on an electronic shaker for 30 min. The absorbance was read with microtitre plate reader (Synergy 2, BioTek Instruments, Inc.) at 540 nm with a reference wavelength of 630 nm. Cell density was calculated as follows:

$$\text{Cell density (\% relative to negative control)} = \frac{\text{As}}{\text{Ac}} \times 100\%$$

where Ac and As represent the absorbance of the average negative control and sample, respectively.

Light microscopy

Cells were cultured in 24-well plates at a density of 2.5×10^4 cells/well overnight before being exposed to 10 and 100 μg/ml extract for 24 h. Plates were viewed with a phase-contrast microscope (Axiovert 40 CFL equipped with a ZEISS AxioCam MRm digital camera) at 10× magnification and at 40× magnification when using polarization–optical transmitted light differential interference contrast (PlasDIC). Morphological features were used to assess cellular status. Apoptotic cells are characterized by cytoplasmic shrinkage, nuclear condensation, membrane blebbing and apoptotic body formation,[27] while necrosis presents with swelling and cell lysis.[27] Pictures were taken and analysed using AxioVision 4 and ImageJ software, respectively.

Live–dead staining

Cells were exposed to extracts as described in the SRB assay. Cells were stained for 5 min with 5 mg/ml fluorescein diacetate (FDA) and 2 mg/ml propidium iodide (PI) staining solution in the dark and washed twice with 200 μl PBS. The PBS was replaced with FCS-free DMEM and images captured with a fluorescence microscope at 10× magnification.

Evaluation of protective effect against oxidative stress

DPPH radical scavenging activity

The DPPH radical scavenging effect of extracts was estimated using the method of Manzocco *et al.*[28] with minor modifications. This assay is based on the principle that the reduction of DPPH by an antioxidant results in a change of colour from purple to yellow which can be measured spectrophotometrically. A solution of 0.135 mM methanolic DPPH was prepared before experimentation. An aliquot of 180 μl DPPH solution was mixed with 20 μl of varying concentrations of the extracts (20–300 μg/ml in reaction), 0.6–20.0 μg/ml Trolox (antioxidant control) or methanol (negative control) in a 96-well plate. The reaction mixture was incubated in the dark for 30 min at room temperature and absorbance read spectrophotometrically at 515 nm using a microplate reader (Synergy-2, BioTek Instruments, Inc.). The ability of

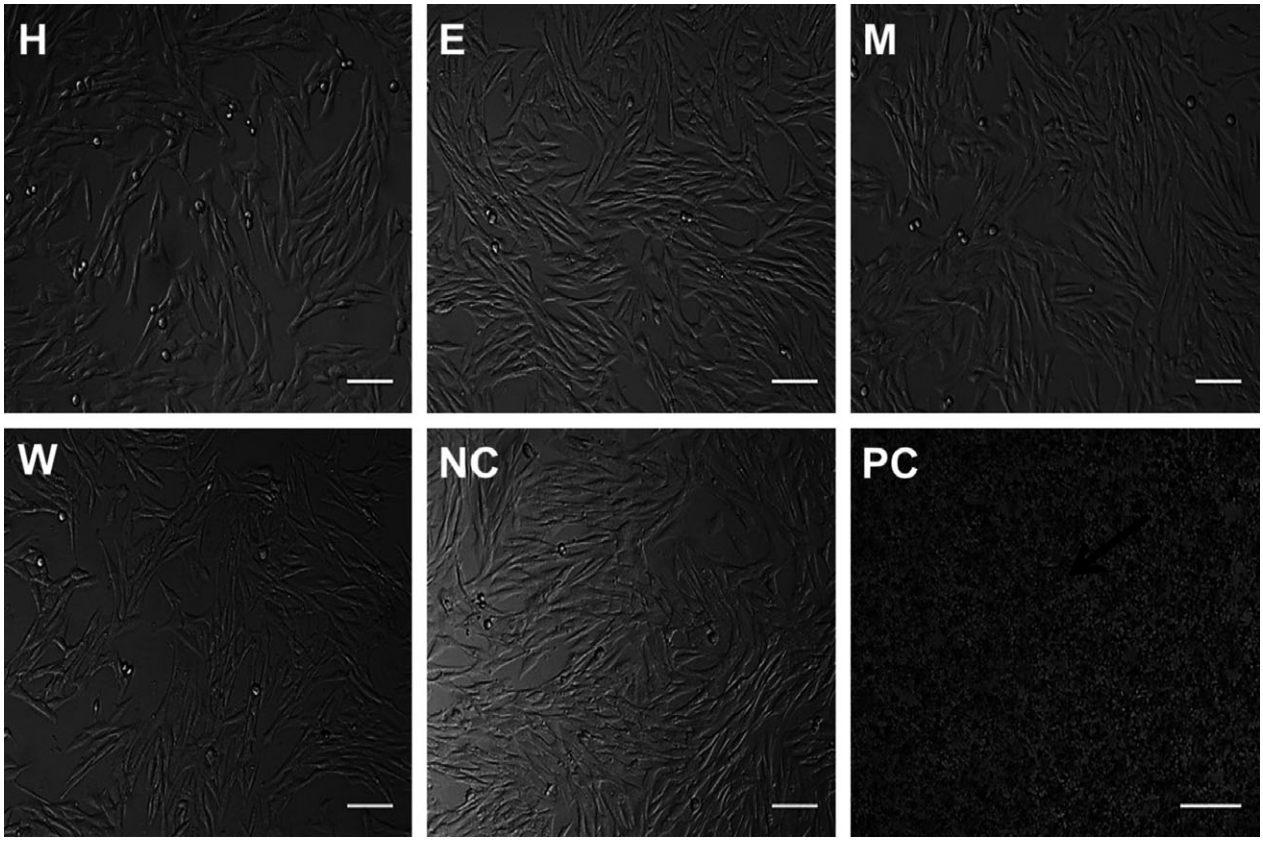


Figure 3 Phase-contrast images of C2C12 myoblasts treated with hexane (H), ethyl acetate (E), methanol (M) and water (W) extracts of *B. diffusa* (100 μg/ml) captured at 10× magnification. Extract-treated cells had normal morphology compared to negative controls (NC) and absence of cellular detachment (black arrow) as seen in the saponin-positive control (PC). Scale bar = 100 μm.

extracts to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH radical scavenging activity}$$
$$(\%\ \text{relative to negative control}) = \left[\frac{\text{Ac} - \text{As}}{\text{Ac}}\right] \times 100$$

where Ac and As represent the absorbance of the average negative control and sample, respectively.

ABTS radical scavenging activity

The 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging effect of extracts was estimated using the method of Re *et al.*[29] This assay is based on the ability of antioxidants to scavenge the stable ABTS radical ($ABTS^+$). The ABTS stock solution, containing 7 mM ABTS salt and 2.4 mM potassium persulfate, was prepared in distilled water and incubated in the dark for 16 h at 4 °C. The resultant $ABTS^+$ solution was diluted with distilled water to an absorbance of 0.70 ± 0.02 at 734 nm (PerkinElmer Lambda 25 UV/VIS spectrometer). An aliquot of 180 μl ABTS+ solution was mixed with 20 μl of varying concentrations of the extracts (20–100 μg/ml in reaction), 0.6–5.0 μg/ml Trolox (antioxidant control) or methanol (negative control) in a 96-well plate. The absorbance was read after 30-min incubation in the dark using the Synergy 2 microplate reader (BioTek Instruments, Inc.). The $ABTS^+$ scavenging capacity was calculated as follows:

$$\text{ABTS radical scavenging activity}$$
$$(\%\ \text{relative to negative control}) = \left[\frac{\text{Ac} - \text{As}}{\text{Ac}}\right] \times 100$$

where Ac and As represent the absorbance of the average negative control and sample, respectively.

Cellular oxidative stress model

Cellular protection was measured in a 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative stress model as described by Ling *et al.*[30] with some minor modifications. The assay is based on the conversion of 2′,7′-dichlorofluorescein diacetate (H_2-DCFH-DA) by

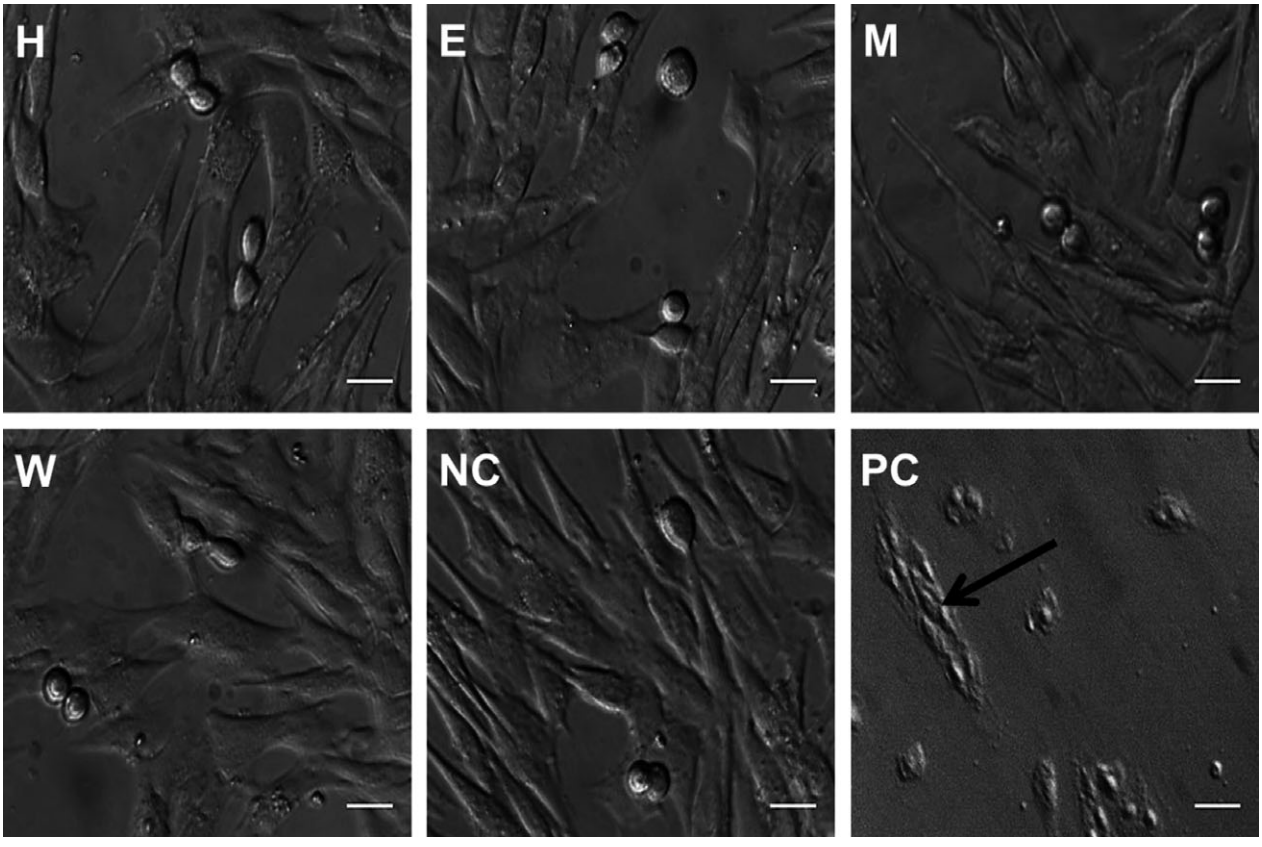


Figure 4 PlasDIC images of C2C12 myoblasts treated with hexane (H), ethyl acetate (E), methanol (M) and water (W) extracts of *B. diffusa* (100 μg/ml) captured at 40× magnification. Extract-treated cells displayed normal morphology compared to the negative controls (NC), with absence of necrosis (black arrow) as observed in the saponin-positive control (PC). Scale bar = 100 μm.

intracellular esterases into the non-fluorescent DCFH, which is subsequently oxidized by ROS to the fluorescent compound DCF.[30] Cells were seeded into a 96-well plate at a density of 1×10^4 cells/well and allowed to attach overnight. The culture media was replaced with 100 μl fresh media containing 10 μM H_2-DCFH-DA and incubated for 30 min in the dark. Excess DCFH-DA was removed by washing twice with 100 μl PBS, followed by exposure to 1, 10 and 100 μg/ml extract for 4 h. All control wells were treated with DMEM during this period, except antioxidant controls to which 5 μg/ml Trolox was added. Cells were washed twice with 100 μl PBS and all wells resuspended in 50 μl PBS containing 100 μM AAPH, with exception of negative controls to which 50 μl PBS was added. The relative fluorescence intensity (RFI) was measured every minute for 2 h at an excitation and emission wavelength of 485 nm and 530 nm, respectively, using a Synergy 2 microplate reader (BioTek Instruments, Inc.). Intracellular ROS was estimated as follows:

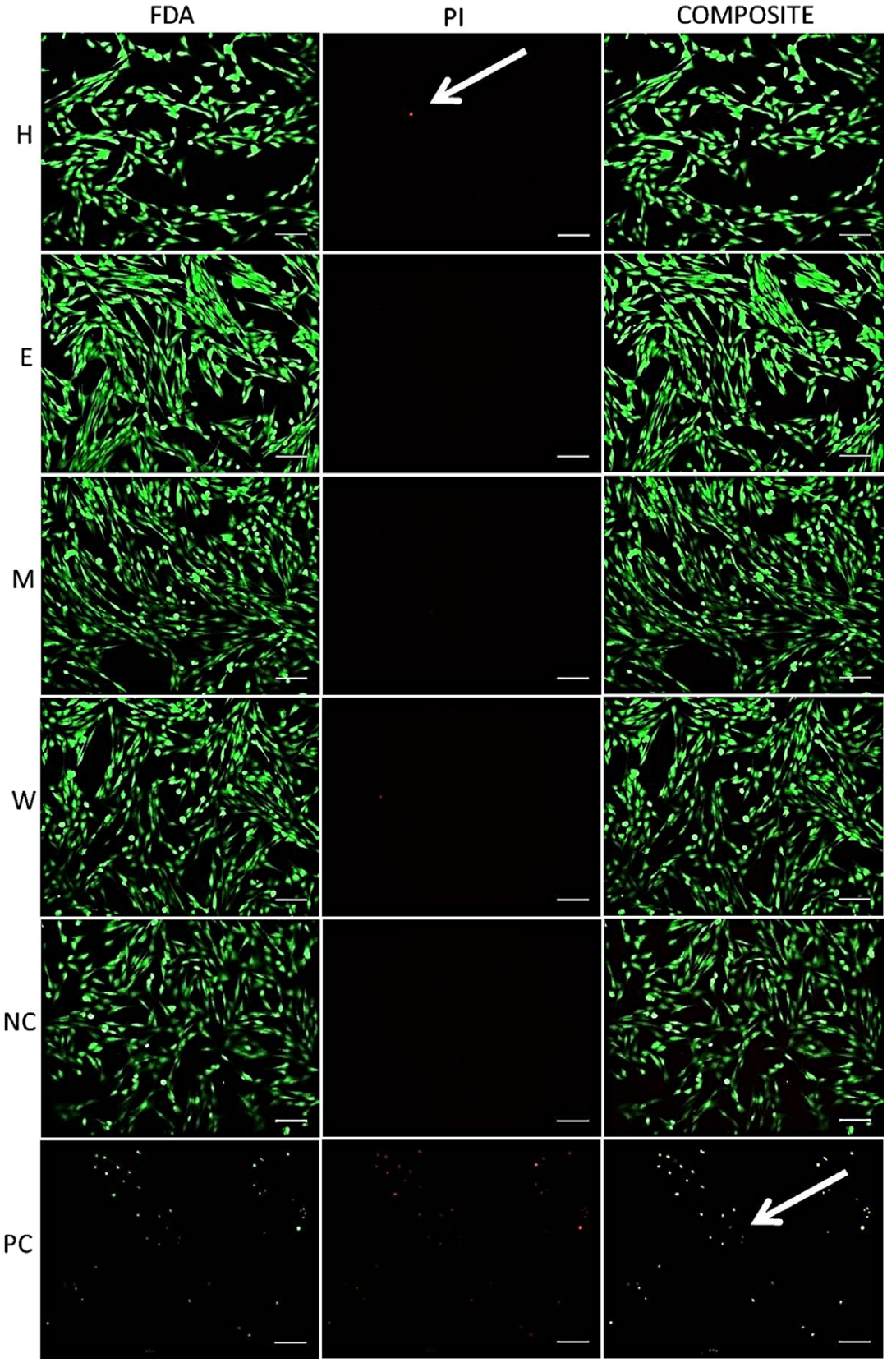
$$\text{Intracellular ROS(relative to control)} = \frac{\text{RFIs}}{\text{RFIc}}$$

where RFIs and RFIc represent the RFI of each sample and the average negative control, respectively.

Evaluation of effect on cellular migration after artificial wound generation

The effect of extracts on cellular migration was assessed using the scratch wound assay as demonstrated by Goetsch and Niesler.[31] This model is based on the observation that upon creation of a new artificial gap (scratch) on a confluent cell monolayer, the cells on the edge of the newly created gap will move towards the opening until cell–cell

Figure 5 Live–dead staining of C2C12 myoblasts treated with hexane (H), ethyl acetate (E), methanol (M) and water (W) extracts of *B. diffusa* (100 μg/ml) captured at 10× magnification. Extract-treated cells were viable (FDA-positive) compared to negative controls (NC), with virtual absence of cells with compromised membrane (PI-negative). The positive control (saponin, PC) displayed compromised membranes (PI-positive, white arrow). FDA: fluorescein diacetate; PI: propidium iodide. Scale bar = 100 μm.


FDA
PI
COMPOSITE
H
E
M
W
NC
PC

contact is re-established.[32] Cells were seeded into 24-well plates at 5×10^4 cells/well and incubated overnight. After incubation, the culture medium was replaced with DMEM supplemented with 0.5% FCS and cultured for a further 24 h to establish a monolayer of cells. A linear wound was generated in the monolayer using a sterile plastic pipette tip, and the percentage wound closure assessed over a period of 24 h in the presence or absence of the plant extracts (10, 100 μg/ml) in triplicates. Digital pictures were taken at 0, 8 and 24 h after creation of the scratches. The wound width was calculated with the aid of image analysis software (ImageJ), and the percentage wound closure calculated as follows:

$$\text{Wound closure(\% relative to wound width at time 0 h)} = \frac{W0 - Wx}{W0} \times 100$$

where W0 and Wx are the wound widths at time 0 h and 8 or 24 h, respectively.

Statistical analysis

Data represent results of at least three independent experiments conducted with technical triplicates. Statistical analysis was performed using GraphPad Prism 5.00 data analysis software. Data were expressed as mean ± standard error of mean (SEM), and difference between groups determined by Kruskal–Wallis test followed by Dunn's post-test (SRB, ROS), as well as two-way ANOVA followed by Bonferroni post hoc tests (scratch assay). *P* values less than 0.05 were considered significant.

Results

Phytochemical screening and analysis

Phytochemical analysis using TLC indicated the presence of alkaloids, saponins, flavonoids, phenols, glycosides, tannins and terpenes (Table 1). A chromatogram of the hexane extract in negative mode is provided in Figure 1. From this analysis, two marker compounds were identified: kaempferol (denoted a) and quercetin (denoted b).

Cytotoxicity evaluation

Sulforhodamine B staining

Extracts did not induce prominent cytotoxicity after 48-h exposure (IC_{50} > 100 μg/ml) (Figure 2). All extracts were generally more cytotoxic within the first 24 h of exposure (Figure 2a), with the cell density of treated cells being higher after 48-h exposure (Figure 2b). The ethyl acetate and methanol extracts were the most and least cytotoxic, reducing cell density by 20.8% and 10.3%, respectively, after 24-h exposure.

Table 2 Antioxidant activity of *B. diffusa* extracts

Treatment	IC_{50} ± SEM (μg/ml)	
	ABTS	DPPH
Hexane extract	>100	>100
Ethyl acetate extract	21.23 ± 1.03	>100
Methanol extract	77.72 ± 1.02	>100
Water extract	>100	>100
Trolox	2.92 ± 1.03	6.27 ± 1.07

Morphological changes

Extract exposure did not induce noticeable morphological changes when compared to the negative controls (Figure 3), although a decrease in cell density was seen in the hexane-treated cells. Furthermore, plasDIC analysis revealed that treated cells had normal cellular membranes and no apparent sign of cytotoxicity (Figure 4).

Fluorescein diacetate staining (green) indicates viable cells, while PI staining (red/orange) indicates loss of membrane integrity. Results from this study demonstrate that treatment with 100 μg/ml of the hexane, ethyl acetate, methanol and water extracts of *B. diffusa* for 24 h had no effect on membrane integrity, as observed effects were compared to untreated controls (Figure 5). The reduced cell density observed in the hexane-treated cells with phase-contrast microscopy was also evident after live–dead staining. No changes were noted after 48-h exposure.

Protective activity against oxidative stress

All extracts displayed greater antioxidant activity against the $ABTS^+$ radical than the DPPH radical (Table 2). The ethyl acetate extract exhibited the highest antioxidant activity with an IC_{50} of 21.23 μg/ml against the $ABTS^+$ radical, followed by the methanol extract (IC_{50} = 77.72 μg/ml). These were, however, lower than the radical scavenging activity of Trolox, which was found to be 2.92 μg/ml and 6.27 μg/ml for the $ABTS^+$ and DPPH radicals, respectively. The IC_{50} recorded in this study was higher than the maximum tested concentration (100 μg/ml) for all extracts against DPPH. The hexane and water extracts also exhibited negligible antioxidant activity against ABTS.

A time-dependent increase of 2.87-fold intracellular ROS was observed upon treatment with 100 μM AAPH compared to the negative control (Figure 6). A dose-dependent suppression of AAPH-induced oxidation was observed upon pretreatment with *B. diffusa* extracts, with the ethyl

acetate and methanol extracts being the most effective treatments. At 100 μg/ml, the ethyl acetate extract reduced intracellular ROS concentrations to 1.58-fold of the negative control ($P < 0.05$), while the methanol extract decreased oxidation by 1.70-fold. Low-dose (1 μg/ml) hexane and ethyl acetate extracts appeared to have an additive effect with AAPH, with intracellular ROS reaching 3.43-fold and 3.18-fold, respectively. Although the later was not significantly different from the positive control, the former was significant ($P < 0.05$).

Effect on cellular migration after artificial wound generation

Treatment with all extracts altered cellular migration, although the effect incurred by the ethyl acetate extract was

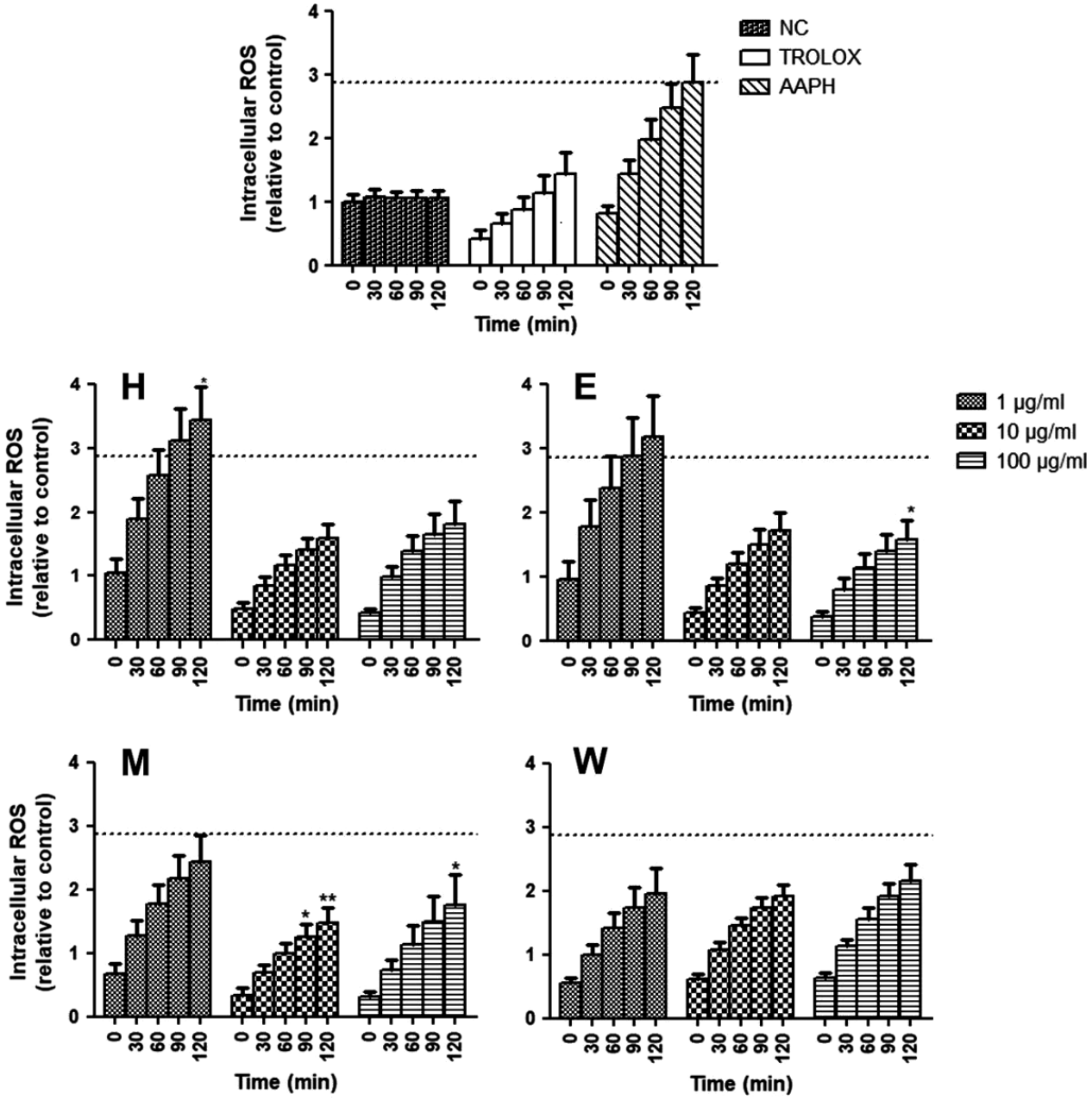


Figure 6 Intracellular ROS concentration of C2C12 myoblast cells treated with hexane (H), ethyl acetate (E), methanol (M) and water (W) extracts of *B. diffusa* (100 μg/ml).The black-dashed line indicate the maximum ROS concentration induced by AAPH at 120 min. Statistical analysis: $*P < 0.05$; $**P < 0.01$ vs. AAPH positive control. NC, negative control.

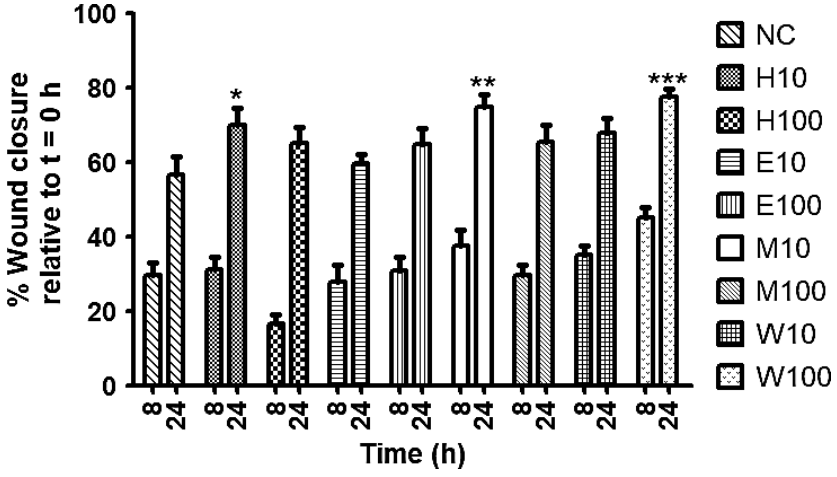


Figure 7 Scratch wound assay conducted on C2C12 myoblasts treated with hexane (H), ethyl acetate (E), methanol (M) and water (W) extracts of *B. diffusa.* Statistical analysis: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. negative control (NC).

not significantly different ($P > 0.05$) from the negative control (Figures 7 and 8). Cellular migration in the hexane-treated and methanol-treated cells was generally higher than the negative controls, although a more prominent effect was observed in the low-dose group (10 μg/ml), with migration being 13.1 and 18.1% higher than the negative controls, respectively. Cells treated with the 10 μg/ml and 100 μg/ml water extract caused an 11.0 and 21.0% increment in cell migration, respectively.

Discussion

Tissue injury can be a major cause of physical disability, with a resultant reduction in quality of life and productivity. Plants have been used for centuries for the management of diverse ailments, including wounds. Phytochemicals are said to modulate various aspects of the healing process by acting as antimicrobials, antioxidants or free radical scavengers, as well as increasing cellular proliferation, migration, angiogenesis, collagen production and DNA synthesis.[33] Pharmacological assessment of *B. diffusa* by various groups of researchers has indicated its activity of antioxidant[12,14,34] and antimicrobial[35,36], which could play a part in its wound healing ability. This study was designed to assess the ability of four sequential extracts of *B. diffusa* to influence oxidation and myoblast wound closure *in vitro*.

The absence of disparity in cell density of treated and untreated cells obtained in this study suggests a lack of notable detrimental effects on myoblast cells. All visualization methods: phase-contrast microscopy, plasDIC and live/dead staining indicated that there were negligible differences between the negative control and the treated cells. The hexane extract, at 100 μg/ml, decreased cell density of treated cells, although other signs of cytotoxicity such as necrosis were absent. This suggests that the hexane extract of *B. diffusa* could have a possible antiproliferative effect. In contrast to treatment with extracts, treatment with saponin resulted in significant cellular damage and death. It has been reported that saponins possess potent cytotoxic activity.[37] The general lack of cytotoxicity observed in this study is confirmed by literature. Apu *et al.*[35] reported negligible *in-vitro* cytotoxicity of the n-hexane, ethyl acetate and methanol extracts of the aerial parts of the plant. An assessment of the ethanol extract showed no increase in cellular volume and protein content in H9C2 cells.[17] The safety of extracts has also been observed *in vivo*.[38,39]

Reactive oxygen species have been identified as one of the key players in the maintenance of cellular physiology by regulating diverse downstream signalling pathways leading to specific functions such as cellular division, growth, apoptosis and necrosis.[8,40,41] However, excessive generation of ROS may result in cellular deterioration.[10] Hence, substances capable of attenuating ROS release could be used as therapeutic options for management of ROS-related medical conditions such as wounds. Pretreatment of C2C12 myoblasts with *B. diffusa* before AAPH-induced oxidation caused a decrease in ROS generation by as much as 1.4-fold compared to the positive controls. Qualitative and quantitative fingerprints of *B. diffusa* have identified phenolic acids and flavonoids, including kaempferol and quercetin.[22] Quercetin and some of its glycosides have exhibited antioxidant activity in a number of studies.[42,43] Therefore, the ROS inhibiting activity could be due to the presence of antioxidant compounds such as those listed above. It is still unclear what might be responsible for the hyperoxidation observed in cells

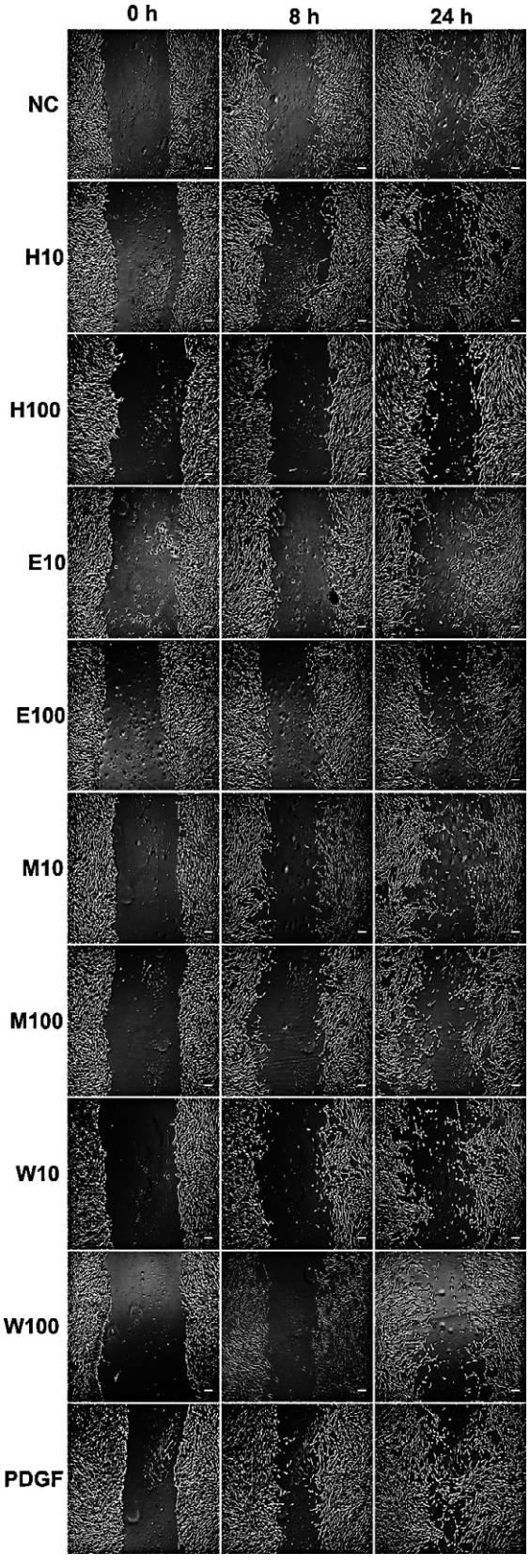


Figure 8 Photomicrographs indicating extent of cellular migration in the scratch wound assay at 0 h, 8 h and 24 h, captured at 5× magnification. PDGF, Platelet-derived growth factor. Scale bar = 100 μm.

pretreated with low-dose hexane and ethyl acetate extracts of *B. diffusa*. It is possible that pre-exposure of cells to very low concentration of antioxidant compounds may have preconditioned the cells before AAPH exposure.[44] Furthermore, some antioxidants such as flavonoids under certain circumstances can act as pro-oxidants, promoting the oxidation of other compounds.[45] There is the possibility of the presence of both antioxidant and pro-oxidant flavonoids in the hexane and ethyl acetate extracts, with the pro-oxidant activity being enhanced at low extract concentrations.

One of the principal components of a healing wound is cellular migration and proliferation, stimulated by factors such as platelet-derived growth factors from inflammatory cells.[46] In this study, the scratch wound assay was used to assess the effect on myoblast migration. To minimize the role of proliferation on the experiment, cells were kept in a lower percentage of serum (0.5%) than those used in the growth media a day before onset of experimentation.[32] This was sufficient to prevent apoptosis or cell detachment, as well as to ensure that any observed effect was a result of exposure to treatment. In this experiment, only the hexane, methanol and water extracts increased myoblast migration significantly. The ROS-mediated oxidation of Akt2 kinase, a signalling molecule that modulates a range of biological processes such as cell survival, proliferation and metabolism, is known to facilitate cellular migration.[47] Therefore, substances that suppress ROS release are expected to slow down cellular migration because of diminished Akt2 kinase activation, while pro-oxidant substances increase migration. The higher myoblast migration observed with lower doses of the hexane and methanol extracts of *B. diffusa* could therefore be due to their minimal potential to inhibit oxidation. This could also explain the effect seen in the water extract treatment, with cellular migration increasing as a result of its inability to suppress oxidation. It is not certain yet which compound(s) are responsible for the observed alteration in cell migration. However, preliminary phytochemical screening has indicated the presence of phenols and flavonoids. Some phenols and flavonoids, including rutin, have been shown to have an effect on wound closure.[22,48] Further studies are, therefore, necessary to determine the compounds responsible for the enhanced cellular migration in *B. diffusa* and to investigate which molecular pathways might be altered.

In conclusion, this study has demonstrated that *B. diffusa* can protect against oxidative stress and promote

wound healing *in vitro* and therefore has potential for therapeutic use in wound treatment. While the hexane and methanol extracts increased wound closure at low concentrations (10 μg/ml), the water extract did so at higher concentration (100 μg/ml). All extracts, however, have indicated a potential to suppress oxidative stress at high concentrations. There is, therefore, need for further experiments to determine effect of the extracts on intracellular pathways such as the Akt2 kinase inhibitory activity. Additionally, there is need to determine the presence of antioxidant and pro-oxidant compounds, as well as others that might be responsible for the observed bioactivity.

Declarations

Conflict of Interest

The authors declare no conflict of interest.

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