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Effect of ethnomedicinal extracts used for wound healing on cellular migration and intracellular reactive oxygen species release in SC-1 fibroblasts

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ABSTRACT

The inhibition of reactive oxygen species (ROS) and the migration of fibroblasts are key processes involved in wound healing. In this study, the ability of aqueous ethnomedicinal plant extracts prepared from *Aspilia africana* CD Adams, *Boerhavia diffusa* L. and *Erythrina senegalensis* DC. to mediate fibroblast migration and ROS release was determined. Phytochemical composition was assessed using thin-layer chromatography (TLC), whereas phytochemical markers were detected using ultra-performance liquid chromatography coupled to time of flight mass spectrometry (UPLC-TOF-MS). Sulforhodamine B staining and morphological examination *via* microscopy was conducted to determine cytotoxic effects on SC-1 fibroblasts. The effect on AAPH-induced oxidative stress was assessed by measuring ROS release using dichlorofluorescein diacetate activation. The scratch wound assay was used to estimate the rate of cellular migration. Alkaloids, flavonoids and phenols were detected in all three extracts using TLC, whilst UPLC-TOF-MS revealed the presence of neobavaisoflavone in *E. senegalensis*. None of the extracts was cytotoxic to the SC-1 cells at the highest in-well concentration tested (100 μg/mL). *E. senegalensis* extract reduced intracellular ROS and cellular migration by 35% and 32.5%, respectively. Although these plant extracts have the potential to minimise oxidation, they do not facilitate fibroblast migration. Further investigation into their mechanism of wound healing is required.

1. Introduction

Healing of wounded skin involves a cascade of complex biological processes which rely on the interaction between several mediators, including platelets, extracellular matrix molecules, fibroblasts, and several other inflammatory cells and products (Martin and Nunan, 2015). Cellular migration is generally pivotal to many physiological and pathological processes, including embryogenesis, angiogenesis, cancer growth and invasion, inflammatory response, and wound healing (Ascione et al., 2016). The proliferation and recruitment of fibroblasts, stimulated by growth factors released by platelets, monocytes and other cellular constituents during the wound healing process, is of particular importance because they are directly responsible for key events in the healing process such as deposition of extracellular matrix components, and collagen (Darby et al., 2014).

The presence of fibroblasts stimulates the production of diverse cytokines related to wound healing, such as interferons (INF)-α, β and γ for macrophage activation, and keratinocyte growth factors for keratinocyte differentiation, proliferation and migration (Broughton et al., 2006). Therefore, substances which affect fibroblast function could affect the healing process. Recently, it has been shown that apart from its antimicrobial effects, silver nanoparticles can also promote wound healing by facilitating fibroblast migration (You et al., 2017). Though much improvement has been made in conventional wound treatment across the world, many patients, particularly in poorly resourced countries have over the years relied on natural products such as medicinal plants for treatment (Pereira and Bartolo, 2016).

Medicinal plants provide a plethora of compounds which could serve as leads for drug discovery. Phytochemicals such as flavonoids and phenolic compounds, amongst a host of others, have been demonstrated to possess useful biological activities such as antioxidant and antimicrobial effects that could account for their use as healing agents (Sasidharan et al., 2011). *Aspilia africana* CD Adams (haemorrhage plant, Asteraceae), *Boerhavia diffusa* L. (spreading hogweed, Nyctaginaceae) and *Erythrina senegalensis* DC (coral flower, Papilionaceae) are examples of commonly used medicinal plants in tropical countries for management

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of several disease states, amongst others, wounds (Okoli et al., 2007; Kuete et al., 2014; Patil and Bhalsing, 2016). All three plants have been shown to possess a diverse array of phytochemicals with multiple pharmacological properties to attest for their ethnomedicinal use (Okoli et al., 2007; Kuete et al., 2014; Patil and Bhalsing, 2016). In a previous study, the wound healing potential of fractions sequentially extracted from *B. diffusa* was demonstrated (Yahaya et al., 2018). The current study determined the ability of aqueous ethnomedicinal plant extracts prepared from *Aspilia africana*, *Boerhavia diffusa* and *Erythrina senegalensis* to effect fibroblast migration and ROS release.

2. Methods

2.1. Plant material

Plant specimens were collected from the University of Cape Coast (UCC) botanical gardens in the Central Region of Ghana, and authenticated by a botanist at the University's School of Biological Sciences herbarium. Voucher specimens for *A. africana* (UCCH 0211215), *B. diffusa* (UCCH 0041215) and *E. senegalensis* (UCCH 0091215) are deposited at the aforementioned herbarium. Plant material was washed, shade-dried at room temperature, and powdered using an electronic grinding device. Powdered samples were stored in air-tight containers until extraction.

2.2. Extract preparation

Powdered plant material (10 g) was extracted by immersion in distilled water (100 mL), agitating on an electronic shaking device for 30 min, and incubating for 24 h at 4 °C. Samples were first filtered through a Whatman Grade-1 filter paper, and subsequently a 0.22-μm Millipore filter paper under vacuum-pressure. The resultant filtrates were freeze-dried (Labconco 31 Freezone 6), and stored at −20 °C until use.

2.3. Chemicals

Dulbeco's modified Eagle's medium (DMEM), foetal calf serum (FCS), penicillin/streptomycin, saponin, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2′,7′-dichlorofluorescein diacetate (H2-DCF-DA), sulforhodamine B (SRB), trichloroacetic acid (TCA), trolox, platelet-derived growth factor (PDGF), and potassium persulfate were purchased from Sigma-Aldrich (South Africa). Phosphate buffered saline (PBS) was acquired from Becton Dickinson (South Africa), whilst tris (hydroxymethyl) aminomethane, and acetic acid were purchased from Merck (South Africa) and Saarchem (South Africa), respectively.

2.4. Phytochemical analysis

The presence of major phytochemical classes in the extracts was determined using thin layer chromatography (Stahl, 1962). Ultra-performance liquid chromatography, coupled to time of flight mass spectrometry (UPLC-TOF-MS), was employed to verify marker compounds in the extracts (Yahaya et al., 2018). UPLC-TOF-MS was conducted on a Waters instrument coupled in tandem to a Waters photodiode array (PDA) detector. A SYNAPT G1 HDMS mass spectrometer was used to generate accurate mass data. MassLynx 4.1 (SCN 872) software was used to control the hyphenated system as well as for data manipulation.

2.5. Cell culture and maintenance

The SC-1 mouse fibroblast cell line was purchased from the American Type Culture Collection (ATTC, CRL-1404), and cultured in DMEM supplemented with 10% FCS, and 1% antibiotic solution (penicillin–streptomycin). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.6. Determination of the effect on cell density

The effect of extracts on cell density was determined using the SRB assay as described previously (Vichai and Kirtikara, 2006). Briefly, cells were seeded into 96-well plates at a density of 1×10^4 cells/well in 10% FCS-supplemented DMEM and incubated for 24 h at the conditions stated above to allow for adherence. Cells were then treated with extracts at final in-well concentrations of 1, 3.2, 10, 32 and 100 μg/mL (prepared in FCS-free DMEM), and incubated for 24 or 48 h. Saponin (1%) and distilled water (0.05%) were used as positive and vehicle controls, respectively. Cells were fixed by adding 50 μL of 50% cold TCA, and incubated at 4 °C overnight. Fixed cells were gently washed with water, dried in a low temperature oven, and stained with 0.057% SRB (in 1% acetic acid) solution for 30 min at room temperature. The plate was then washed three times with 200 μL of 1% acetic acid solution to remove excess dye, and dried. The bound dye was dissociated in 200 μL of a 10 mM Tris-base solution (pH 10), and the absorbance measured at 540 nm using a BioTek ELx800 microplate reader. Cell density was determined using the following equation:

$$\textit{Cell density}\ (\%\textit{of the negative control}) = \frac{As}{Ac} \times 100\%$$

where *As* and *Ac* are the blank-corrected absorbances of sample and average negative control respectively.

2.7. Morphological studies

Cell morphology was examined using phase contrast and plasDIC microscopy. Treatments for the morphological studies were similar to that described in Section 2.6, except that cells (2.5×10^4 cells/well) were exposed to the highest concentration of extract tested (100 μg/mL) in 24-well plates. Following 24 and 48 h incubation at the conditions stated above, cells were examined microscopically and pictures taken at 10× (phase contrast) and 40× (plasDIC) magnification.

2.8. Assessment of the effect on oxidative stress

2.8.1. Antioxidant activity determination

The 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging effect of extracts was estimated using a previously described method (Re et al., 1999). 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 (in-reaction, 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.) at 734 nm. The $ABTS^+$ scavenging capacity was calculated as follows:

$$\textit{ABTS radical scavenging activity}\ (\%\textit{relative to negative control}) = \frac{Ac - As}{As} \times 100$$

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

2.8.2. Effect on AAPH-induced oxidative stress

The ability of extracts to alter intracellular ROS was assessed in an AAPH-induced oxidative stress model as described previously (Lopachev et al., 2016) with minor modifications. Cells were seeded into a 96-well plate at a density of 1×10^4 cells/well and allowed to attach overnight. The culture medium was replaced with 100-μL fresh medium containing 10-μM H2-DCFH-DA and incubated for 30 min in the dark. Excess H2-DCF-DA was removed by washing twice with 100-μL PBS, followed by exposure to 1, 10 and 100-μg/mL extract (in 100 μL of 10% FCS-supplemented DMEM) 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 re-suspended in 50-μL PBS containing 100-μM AAPH, with the exception of the negative controls to which 50-μL PBS was added. The relative fluorescence intensity (RFI) was measured every min 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:

$$\textit{Intracellular ROS}\ (\textit{fold}-\textit{change relative to control}) = \frac{RFIs}{RFIc}$$

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

2.9. The effect on cell migration

Effect on fibroblast cell migration was assessed *in vitro* using the scratch wound assay (Liang et al., 2007). Cells were seeded into 24-well plates as described in Section 2.7 in 10% FCS-supplemented DMEM, and incubated for 24 h to allow for cellular attachment. Attached cells were gently washed with PBS, the medium replaced with 0.5% FCS-supplemented DMEM, and incubated for a further 24 h to form a monolayer. An intersecting vertical and horizontal scratch was generated in the cell monolayer of each well, with the latter serving as a reference point for image acquisition. Cellular debris was removed by washing with PBS. Cells were then exposed to extracts at concentrations of 10 and 100 μg/mL (in-reaction) in 0.5% FCS-supplemented DMEM. PDGF (2 ng/mL in-reaction) served as positive control. The scratches were microscopically examined and pictures taken at 0, 8, and 24 h after wound generation. The average wound area was estimated using the ImageJ software, and cell migration calculated as:

$$\textit{Cell migration}\ (\%) = \frac{Ao-At}{Ao} \times 100\%$$

where *Ao* and *At* represent average wound area at 0 h and 24 h respectively.

2.10. Statistical analysis

Data represents results of at least three independent experiments conducted in technical triplicates. Statistical analysis was performed using GraphPad Prism 5.00 data analysis software. Data was expressed as the mean ± standard error of mean (SEM). Non-linear regression was used to determine half-maximal inhibitory concentrations (IC50). The difference between groups was determined by a Kruskal–Wallis test followed by Dunn's post-hoc test (cytotoxicity and ROS), as well as two-way ANOVA followed by Bonferroni post-hoc tests (scratch assay). *P* values less than 0.05 were considered significant.

3. Results and discussion

3.1. Phytochemical evaluation

Phytochemical composition of the extracts, using TLC, is represented in Table 1. All the extracts contained major phytochemical groups such as alkaloids, flavonoids and phenols. These findings are also reported in previous literature (Saidu et al., 2000; Okwu and Josiah, 2006; Sreeja and Sreeja, 2009).

Neobavaisoflavone was detected in the *E. senegalensis* extract using UPLC-TOF-MS (Fig. 1). The presence of three cytotoxic isoflavonoids, including neobavaisoflavone, has been reported in the plant (Kuete et al., 2014). Ascorbic acid and kaempferol were not detected, although they had previously been reported in methanol extracts of *A. africana*, and *B. diffusa*, respectively (Ferreres et al., 2005; Okwu and Josiah, 2006). The lack of detection of these compounds could be ascribed to differing extraction solvents employed or geographical variation of harvested plants.

3.2. Cytotoxicity

Cellular density of fibroblasts treated with the extracts at varying concentrations (up to 100 μg/mL) resulted in a maximum of 21% death after 24-h and 48-h exposure (Fig. 2). The *E. senegalensis* extract was the most cytotoxic to the SC-1 fibroblasts, with a reduction in cellular density of 9% and 21% after 24-h (Fig. 2A) and 48-h (Fig. 2B) exposure, respectively. Furthermore, evaluation using phase contrast (Fig. 3A) and plasDIC microscopy (Fig. 3B) indicated normal morphology after treatment with the plant extracts, in contrast to the positive control, saponin, where cytotoxicity occurred.

Negligible cytotoxicity (IC_{50} > 300 μg/mL) has been reported for the hydroalcoholic extract of *B. diffusa* in drug-sensitive human T-lymphoblastoid (CCRF-CEM) and multidrug-resistant P-glycoprotein over-expressing leukaemia (CEM/ADR5000) cell lines (Tacchini et al., 2015). The isoflavones, neobavaisoflavone, sigmoidin H and isoneorautenol, have been reported to be cytotoxic (Kuete et al., 2014). These compounds could be responsible for the higher cytotoxic activity noted in this study for the *E. senegalensis* extract.

3.3. Effect on oxidation

The extract with the highest $ABTS^+$ radical scavenging activity was *A. africana* (IC_{50} = 36.18 μg/mL), compared to an IC_{50} of 51.52 μg/mL for *E. senegalensis* and more than a 100 μg/mL for *B. diffusa*. The positive control, Trolox, had an IC_{50} of 2.92 μg/mL. Pre-treatment of cells with 100-μg/mL *E. senegalensis* and *A. africana* extracts prior to induction of oxidative stress led to a 35% and 25% reduction in ROS release, respectively (Fig. 4). *E. senegalensis* (1 μg/mL) was better at suppressing ROS, even though *A. africana* had a higher $ABTS^+$ scavenging activity. This suggests a probable diminished uptake of *A. africana* into the cells, or that a different mechanism of quenching is at play. Although the response was not dose-dependent, differences between the various

Table 1
Presence of Phytochemical classes in the plants investigated in this study as determined by thin layer chromatography.

Extract	Phytochemical classes						
	Alkaloids	Flavonoids	Glycosides	Phenols	Saponins	Tannins	Terpenes
Aspilia africana	+	+	−	+	−	−	+
Boerhavia diffusa	+	+	+	+	−	−	−
Erythrina senegalensis	+	+	+	+	−	−	+

+ = presence of component; − = absence of component.

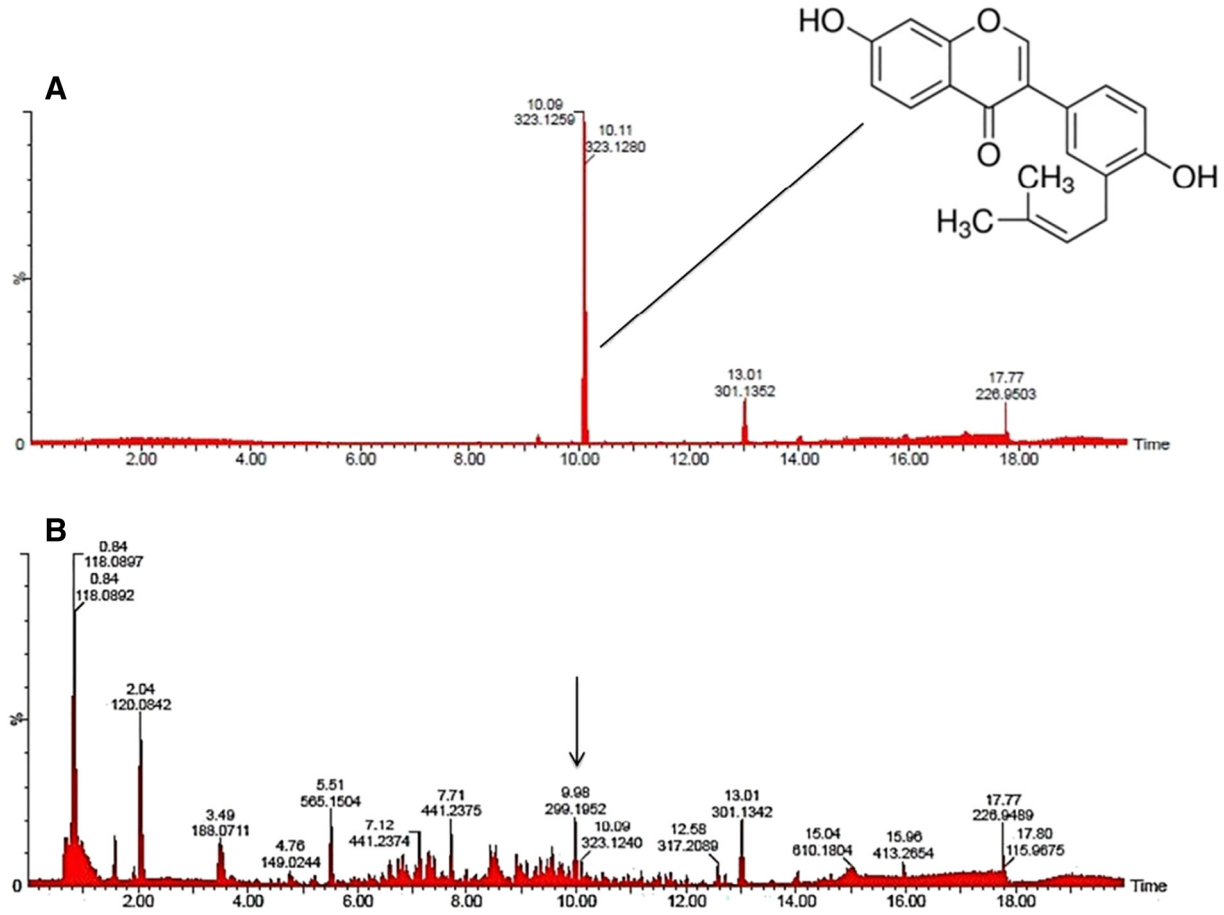

Fig. 1. UPLC-TOF-MS spectra of neobavaisoflavone (A) and *Erythrina senegalensis* ethnomedicinal extract. (B) The arrow indicates the presence of neobavaisoflavone.

concentrations tested was not statistically significant. The ethnomedicinal extract of *B. diffusa* on the other hand had no effect on the amount of ROS released, which confirms results obtained in the $ABTS^{+}$ scavenging assay.

Reactive oxygen species are crucial for the maintenance of normal cellular physiology in that they regulate signalling pathways which control cell growth, metabolic rate, cell division, apoptosis, necrosis and ageing (Menon and Goswami, 2007). Therefore, an optimal amount of ROS results in timeous healing of wounds. Uncontrolled ROS release or depletion of intracellular antioxidants results in oxidative stress, which delays healing by damaging cells, tissues and other components in the body (Menon and Goswami, 2007). The ability of the extracts to suppress oxidative stress could be useful in managing diseases where oxidative stress is involved such as in delayed wound healing. Phytochemical components, such as the flavonol kaempferol, have been shown to inhibit ROS (Wang et al., 2006). Further studies are required to identify the compounds responsible for this activity.

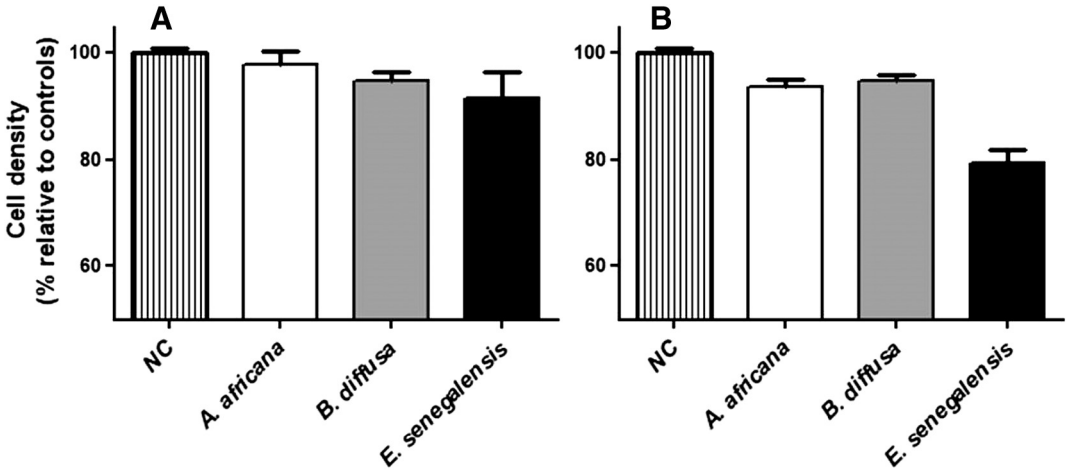

Fig. 2. Effect of 100 μg/mL of the ethnomedicinal extracts on fibroblast cell density after (A) 24-h and (B) 48-h exposure. NC: Negative control.

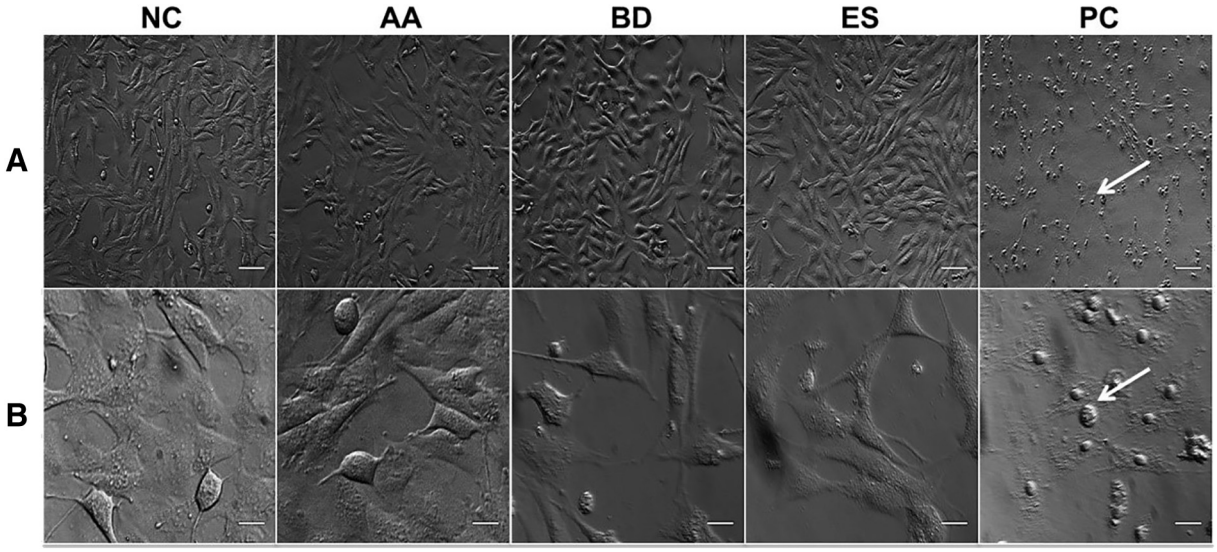

Fig. 3. The effect of ethnomedicinal extracts on SC-1 fibroblast morphology. Images were captured by (A) phase contrast at 10× magnification and (B) plasDIC at 40× magnification. AA: *A. africana*; BD: *B. diffusa*; ES: *E. senegalensis*; NC: Negative control; PC: positive control. Arrows indicate damaged cells. Scale bar: 100 μm.

3.4. Fibroblast migration

Cellular migration is an important process in wound repair, allowing fibroblasts to traverse local tissue environments in order to degrade, repair and remodel the extracellular matrix (Tschumperlin, 2013). Twenty four hours after artificial wound generation, none of the extracts were found to have facilitated wound closure when compared to the negative control (Figs. 5 and 6). Wound closure in the negative control cells was 64.4%, compared to 80% in the positive control (PDGF). Treatment with all three ethnomedicinal extracts decreased fibroblast migration relative to the negative control, particularly at high concentrations (100 μg/mL). Though 10-μg/mL *A. africana* extract had no effect on cellular migration, 100 μg/mL hindered migration by 5.2%. Treatment with *B. diffusa* extract on the other hand produced a 7.6% and 21.8% suppression of cell migration at 10 μg/mL and 100 μg/mL, respectively compared to the negative control. The *E. senegalensis* extract had the greatest effect on fibroblast migration, with a 16.2% and 32.5% reduction in migration at 10 μg/mL and 100 μg/mL, relative to the negative control respectively.

The observed effect could be linked to the ROS inhibitory ability of the extracts. Whilst extracts with a higher ability to suppress oxidation such as *E. senegalensis* delayed migration to the greatest extent, those with minimal effect on oxidation recorded a much lower inhibitory effect. Cellular migration is regulated by numerous intracellular factors. Inhibition of either the ERK1/2 or p38 pathways resulted in delayed corneal epithelial wound healing (Sharma et al., 2003). Furthermore, activation of the P13K/Akt/mTOR pathway has resulted in accelerated wound closure (Squarize et al., 2010). More recently, oxidation of Akt2 kinase has been shown to facilitate cellular migration (Wani et al., 2011). These suggest a probable relationship between ROS and cellular migration. Therefore, the extracts could be inhibiting migration by indirectly inhibiting the P13K/Akt/mTOR pathway through ROS inhibition.

4. Conclusions

Results obtained in this study have shown that the ethnomedicinal extracts prepared from the three wound healing plant extracts are not cytotoxic in SC-1 fibroblasts at the concentrations tested. Though the *E. senegalensis* extract demonstrated ability to suppress AAPH-induced oxidation, the other two extracts had no effect on ROS release. This could be due to *E. senegalensis'* better antioxidant potential, suggesting

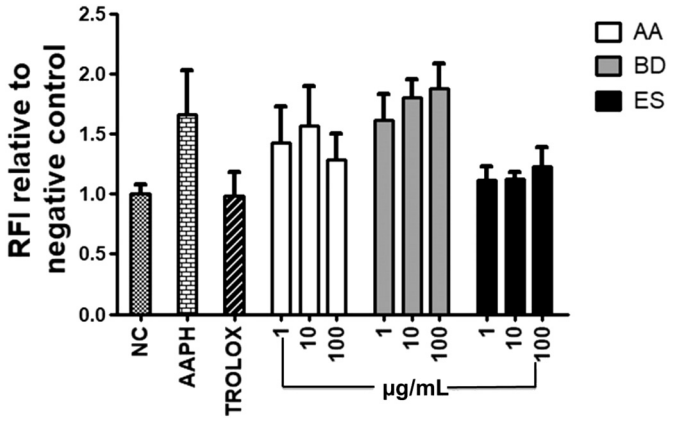

Fig. 4. The effect of ethnomedicinal extracts on ROS release in SC-1 fibroblasts. AA: *A. africana*; BD: *B. diffusa*; ES: *E. senegalensis*; NC: negative control; AAPH: oxidant control; Trolox: antioxidant control.

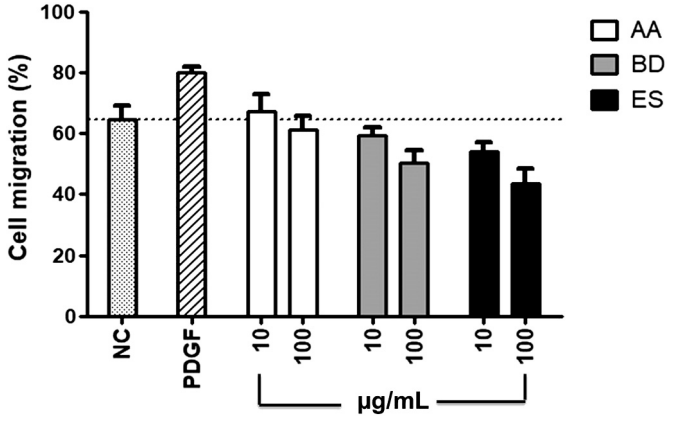

Fig. 5. The effect of ethnomedicinal extracts on SC-1 fibroblast migration over a 24 h period assessed using the scratch wound assay. AA: *A. africana*; BD: *B. diffusa*; ES: *E. senegalensis*; NC: negative control; PDGF: platelet-derived growth factor. Dotted line depicts maximum wound closure in NC.

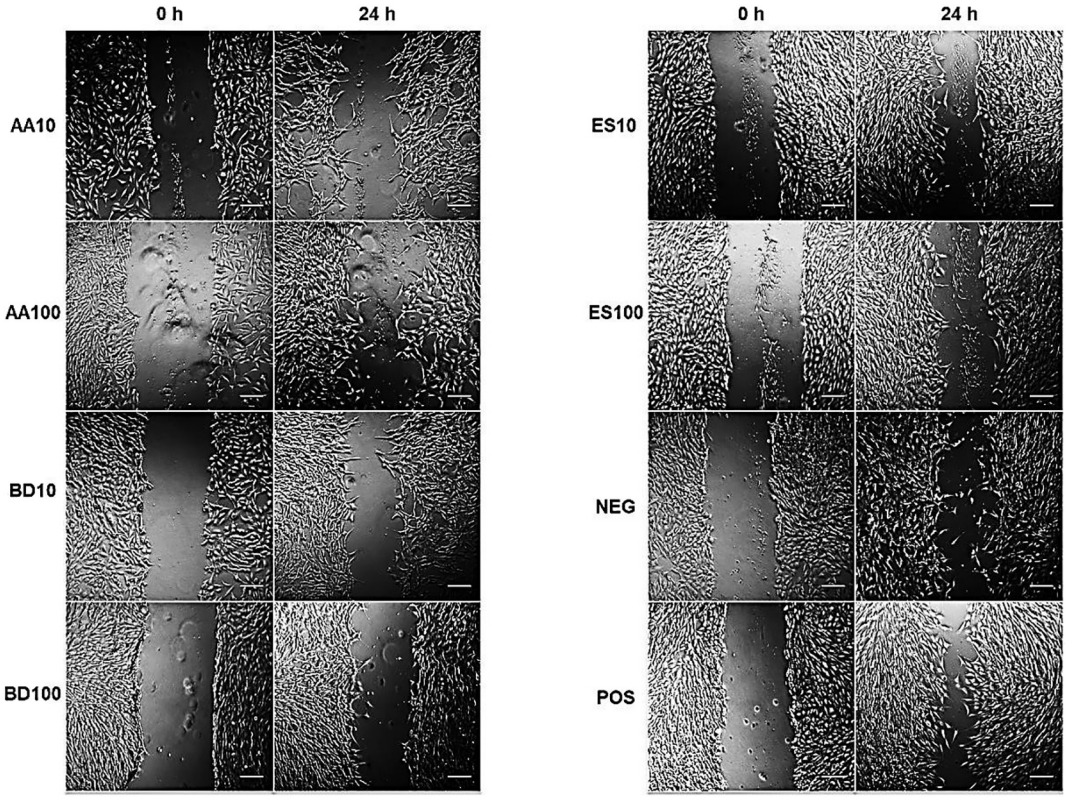

Fig. 6. Micrographs of fibroblast migration after treatment with 10 and 100-μg/mL extracts. AA: *A. africana*; BD: *B. diffusa*; ES: *E. senegalensis*; NC: negative control; PDGF: platelet-derived growth factor. Scale bar = 200 μm.

that it could be useful in the management of ROS-induced chronic wounds. All the extracts however showed tendency to inhibit fibroblast migration, particularly at higher doses. Future studies should be focused at identifying the compounds responsible for these activities, as well as their molecular mechanisms of action.

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