The effect of three Ghanaian plants on fibroblast migration, inflammation and bacterial growth *in vitro*

by

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"To God be all the glory. Great things He has done, and greater things He will do"

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Abstract

Medical conditions which cause morbidity and mortality, such as chronic wounds and infection, lead to significant medical costs. Much of the world's population is dependent on alternative medicine, of which herbal medicine forms a crucial part. In Ghana and other countries around the world, an estimated 70% of the population rely on alternative treatments like herbs for management of various forms of disease. However, even though medicinal plants are widely used for treatment, most have not been scientifically proven to be safe and efficacious. Hence there is need to assess the biological activity of these plants which may be a potential lead in drug development.

This study assessed the wound-related biological activities of three commonly used medicinal plants in Ghana (*Aspilia africana*, *Boerhavia diffusa*, and *Erythrina senegalensis*). Sequential extracts were prepared from the three plants using hexane, ethyl acetate, methanol, and water as solvents, in increasing polarity. Also, ethnomedicinal extracts were obtained with water as solvent, in accordance with the method used by traditional healers. Extracts were screened for phytochemical components using thin layer chromatography (TLC), and phytochemical fingerprinting performed with ultra performance liquid chromatography in tandem with time-of-flight mass spectrometry (UPLC-qTOF-MS). Cytotoxic potential of the extracts in SC-1 fibroblasts, C2C12-myoblasts, and differentiated THP-1-macrophages was determined using the sulforhodamine B staining assay, and cells morphologically assessed with phase contrast, PlasDIC, and live/dead staining microscopy. Acellular antioxidant activity was conducted by exploring the 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid] (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, whilst ability to protect against cellular oxidative stress was assessed using 2',7'-dichlorofluorescin diacetate as marker. Anti-inflammatory potential of extracts was evaluated using xanthine oxidase activity. Also, the ability of extracts to alter closure of artificially generated wounds in fibroblast and myoblast monocultures was determined using the scratch assay. The extracts were also assessed for their antibacterial effect using the disk diffusion and microdilution assays. Extracts with a minimum inhibitory concentration (MIC) above or equal to 1 mg/mL were evaluated for their ability to inhibit bacterial biofilms.

Analysis using TLC indicated that alkaloids, flavonoids, and phenols were the major groups present. The UPLC-qTOF-MS analysis led to the verification of previously identified, as well as tentative identification of already-described phytochemical compounds or their derivatives in the plants. In addition to the already reported ascorbic acid, quercetin was also identified in *A. africana*. Furthermore, kaempferol, quercetin, and rutin were identified in the extracts of *B. diffusa*, the latter compound being identified for the first time in this plant. Also, for the first time, kaempferol, rotenone and rutin, were identified in extracts of *E. senegalensis*. Apart from these three compounds, neobavaisoflavone was also detected.

All the extracts recorded a half maximal inhibitory concentration (IC_{50}) above 100 µg/mL in all three cell-lines. The most cytotoxic extracts to the myoblasts, fibroblasts, and macrophages, were the methanol and ethnomedicinal extracts of *A. africana*, and the hexane extract of *E. senegalensis*, with a maximum of 38.8% reduction in cell density respectively. The minimal cytotoxic potential of the extracts was further confirmed by the absence of morphological differences between treated and untreated cells.

Most of the extracts exhibited good ABTS radical scavenging activity (IC_{50} < 100 µg/mL). The strongest effect against the free radical was observed with the ethyl acetate extract of *B. diffusa* (IC_{50} = 21.23 µg/mL). On the contrary, most of the extracts recorded poor ability to scavenge the DPPH free radical. Only the methanol extracts of *A. africana* (IC_{50} = 278 µg/mL) and *E. senegalensis* (IC_{50} = 291 µg/mL) yielded IC_{50} values below the maximum tested concentration (320 µg/mL). This could possibly be ascribed to the differences in the stereoselectivity between the two free radicals, and the poor DPPH scavenging ability of hydrophilic antioxidants.

The effect of the extracts against AAPH-induced oxidation in the cells correlated with the antioxidant potential of the extracts. Whilst most of the extracts with good antioxidant potential suppressed AAPH-induced oxidative stress, the most profound effect was observed with pre-treatment of macrophages with the ethyl acetate extract of *A. africana*. The extract caused a 1.74-fold decrease in intracellular reactive oxygen species (ROS) concentration after 120 min following pre-treatment with 100 µg/mL, when compared with the AAPH control. This was comparable to the 1.89-fold

reduction caused by the positive control compound, 5 µg/mL Trolox. The ethnomedicinal extracts of *B. diffusa* and *E. senegalensis* exhibited a dose-dependent increase in intracellular ROS in fibroblasts, with intracellular ROS concentration upon treatment with the extracts at 100 µg/mL being at least 23% higher than the negative controls. This suggest that the extracts could exhibit a possible pro-oxidant effect at higher concentrations. Quercetin, a compound with pro-oxidant effects at higher concentrations, was detected in the ethyl acetate extract of *B. diffusa*, which may describe this effect. However, none of the extracts used in the current study demonstrated the ability to significantly inhibit xanthine oxidase activity. The strongest activity against the enzyme (maximum of 15% inhibition) was exhibited by extracts of *E. senegalensis*.

The hexane extract of *A. africana* and the water extracts of *B. diffusa* increased migration of myoblast cells by 44.4% and 39.4%, respectively. This indicates a possible role of the extracts in enhancing collagen deposition and wound remodelling, two processes with myoblast involvement. On the other hand, six of the extracts decreased fibroblast migration, and therefore could have negative effects on wound healing processes such as collagen and matrix metalloproteinase synthesis. Further analysis would be required to ascertain the extent to which the extracts could impact activity of the cells. Also, the methanol extract of *E. senegalensis* (MIC = 0.5 mg/mL in *E. coli*) was the most effective against the micro-organisms tested. All the other extracts had MICs above 1 mg/mL. None of the extracts showed activity against *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and bacterial biofilms.

In conclusion, this study has scientifically demonstrated that the three plants may assist wound healing at different stages in the healing process. This could be achieved through their antioxidant effects, ability to suppress oxidative stress, antibacterial activity, and ability to enhance activity of fibroblasts and myoblasts. Practitioners should be cautioned against using high concentrations because of possible cytotoxicity.

Study outputs

Published articles (Appendix I)

1. **Yahaya ES**, Cordier W, Steenkamp PA, Steenkamp V. Effect of ethnomedicinal extracts for wound healing on cellular migration and intracellular reactive oxygen species release in SC-1 fibroblasts. South African Journal of Botany. 2018; 118:11-17.
2. **Yahaya ES**, Cordier W, Steenkamp PA, Steenkamp V. Attenuation of oxidative stress and artificial wound closure in C2C12 myoblasts induced by sequential extracts of *Boerhavia diffusa*. Journal of Pharmacy and Pharmacology. 2018;70(1):111-23.

Published conference abstract

Yahaya ES, Cordier W, Steenkamp V. Comparative Effect of three wound healing ethnomedicinal extracts on fibroblast migration and reactive oxygen species release *in vitro*. Journal of Innovation and Research in Health Sciences & Biotechnology. 2017; 2(4) Suppl: 1-26

Article(s) under review

Gulumian M, **Yahaya ES**, Steenkamp V. African herbal remedies with antioxidant activity: A potential resource base for wound treatment? Evidence-Based Complementary and Alternative Medicine. Submitted in May 2018

Conference presentations

A. International conferences

1. **Yahaya ES**, Cordier W, Steenkamp PA, Steenkamp V. Ethnomedicinal extracts of three Ghanaian plants attenuate oxidation in SC-1 fibroblasts, while perturbing cellular migration. World Congress of Pharmacology, Kyoto, Japan. 1st – 7th July 2018 (**Poster presentation**)
2. **Yahaya ES**, Cordier W, Steenkamp PA, Steenkamp V."Modulation of intracellular reactive oxygen species release and wound closure of C2C12 myoblasts induced by sequential extracts of *Boerhavia diffusa*. Society for Medicinal Plants and Economic Development Conference, Johannesburg, South Africa. 27th – 30th August 2017 (**Oral presentation**)

3. **Yahaya ES**, Cordier W, Steenkamp PA, Steenkamp V. Comparative effect of three wound healing ethnomedicinal extracts on fibroblast migration and reactive oxygen species release *in vitro.* 1st International Conference on Clinical Trial and Innovative Therapeutics, Cape Town, South Africa. 24th – 26th July 2017 (**Oral presentation**)

B. National presentations

1. **Yahaya ES**, Cordier W, Steenkamp PA, Steenkamp V. "*In vitro* characterization of the wound-related bioactivities of extracts from three plants of Ghanaian origin. 1st Conference of Biomedical and Natural Sciences and Therapeutics, Spier Estate, Stellenbosch, South Africa. 7th – 10th October 2018. **(Oral presentation)**
2. **Yahaya ES**, Cordier W, Steenkamp PA, Steenkamp V. Ethnomedicinal extracts of three Ghanaian plants attenuate oxidation in SC-1 fibroblasts, while perturbing cellular migration. Health Sciences Faculty Day, University of Pretoria, South Africa. 21st – 22nd August 2018. **(Oral presentation)**
3. **Yahaya ES**, Cordier W, Steenkamp PA, Steenkamp V. Modulation of intracellular reactive oxygen species release and wound closure of C2C12 myoblasts induced by sequential extracts of *Boerhavia diffusa*. Health Sciences Faculty Day, University of Pretoria, South Africa. 22nd – 23rd August 2017. **[Awarded 2nd Place: Oral presentations, Basic medical sciences category]**

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List of abbreviations

ºC	Degree Celsius
%	Percentage
α	Alpha
β	Beta
µg/mL	Microgram per milliliter
µL	Microliter
µm	Micrometer
µM	Micromolar
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
β-CLAMS	β-carotene-linoleic acid model system
BrdU	5-Bromo-2'-deoxyuridine
CC	Column chromatography
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
Cu	Copper
DCF	2,7-Dichlorofluorescein
DIC	Differential inference contrast
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPS	Extracellular polysaccharide matrix
ESI	Electro spray ionization

EU	European Union
FCS	Foetal calf serum
FDA	Fluorescein diacetate
Fe	Iron
FGF	Fibroblast growth factor
FRAP	Ferric-reducing antioxidant power
FTIR	Fourier-transform infrared spectroscopy
GC	Gas chromatography
GM-CSF	Granulocyte macrophage-colony stimulating factor
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HDMS	High definition mass spectrometry
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
IL	Interleukin
INF	Interferon
INT	Iodonitrotetrazolium
IV	Intravenous
KGF	Keratinocyte growth factor
LC	Liquid chromatography
LCMS	Liquid chromatography mass spectrometry
LMWH	Low molecular weight heparin
m/z	Mass to charge ratio
MBC	Minimum bactericidal concentration
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
MLP	Microsomal lipid peroxidation
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MRSA	Methicillin-resistant *Staphylococcus aureus*

MS	Mass spectrometry
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NADPH	Reduced nicotinamide dinucleotide phosphate
NHS	National Health Service
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2	Oxygen
O_2•	Superoxide anion
OH•	Hydroxyl radical
$ONOO^-$	Peroxynitrite
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBP	Penicillin-binding protein
PC	Paper chromatography
PCNA	Proliferating cell nuclear antigen
PDA	Photodiode array
PDGF	Platelet-derived growth factor
PF	Platelet factor
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
PO	Per os
Prx	Peroxiredoxin
PTT	Partial thromboplastin time
Rf	Retention factor
RFI	Relative fluorescence intensity
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute

RT	Room temperature
SA	South Africa
SAHPRA	South African Health Products Regulatory Authority
SEM	Standard error of mean
SOD	Superoxide dismutase
SRB	Sulforhodamine B
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TEAC	Trolox equivalence antioxidant capacity
TGF	Transforming growth factor
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
TOF	Time-of-flight
TPP	Total polyphenol
Trx	Thioredoxin
TTC	2,3,5-Triphenyl-2H-tetrazolium chloride
UK	United Kingdom
UPLC	Ultra-performance liquid chromatography
USA	United States of America
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
v/v	Volume per volume
WHO	World Health Organisation
WT-1	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium
XO	Xanthine oxidase
XTT	2, 3 Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

Chapter 1

General introduction

1.1. The burden and pathophysiology of wounds

Wounds are physical injuries that result when the skin is broken or an opening occurs, with subsequent disturbance of its anatomy and function.[1] The result is a loss of continuity of epithelium with or without the loss of underlying connective tissue. Badly treated or untreated wounds can become chronic and represent a significant burden in patients due to cost and duration of treatment.[2]

Wounds are a major cause of morbidity and mortality, placing a financial burden on both the patient and healthcare systems.[2] In the United Kingdom alone, an estimated 2.2 million wound cases were reported in 2012 and 2013 according to National Health Service (NHS) records.[2] The annual cost to the NHS varied between £1·94 billion (for managing 731 000 leg ulcers) and £89·6 million (for managing 87 000 burns and associated comorbidities). Furthermore, the cost of treating a chronic wound was on average 135% higher than that of an acute wound.[2] Statistics on the occurrence and management of wounds in Africa are scarce. In Nigeria, wounds constitute 32% of all hospital admissions, and accounts for 14.3% of all mortality and disability-adjusted life years in South Africa.[3, 4] Patients and healthcare systems could therefore benefit from strategies that are focused towards prevention and identification of treatments that promote effective healing of wounds.

Virtually every tissue and organ within the body is capable of healing or repairing itself after injury. Wound healing is a complex process which results in the skin or other organ tissue repairing itself after injury. A set of biochemical events take place in a closely ordained cascade to repair the damage caused by injury.[1] Within minutes of injury, platelets (thrombocytes) aggregate at the injury site to form a fibrin clot which acts to control active bleeding and maintain haemostasis.[5] Under normal circumstances, wounds follow the conventional stages of healing and are healed within a short period of time.[5]

However, non-healing or chronic wounds (Figure 1), may remain unhealed for many months thereby undermining the health and general wellbeing of individuals.[1, 5] These are wounds in which the normal process of healing is altered at one or more points of the healing process. Among factors which predispose individuals to chronic wounds are changes in profile and activity of cells, altered deposition of extracellular matrix components, failure of epithelialisation, and elevated levels of free radicals and microorganisms.[5, 6] Also, conditions such as diabetes, chronic renal failure, arterial or venous insufficiency, and radiation-induced tissue damage are among the multifactorial processes that further contribute significantly to dysfunctional wound healing.[7, 8] These conditions lead to the alteration of the inflammatory response, causing dysfunctional activities of macrophages. Consequently, the ability to combat infection is limited, angiogenesis and vasculogenesis is impaired, fibrotic tissue accumulates, and aberrant extracellular matrix deposition occurs.[6, 9]

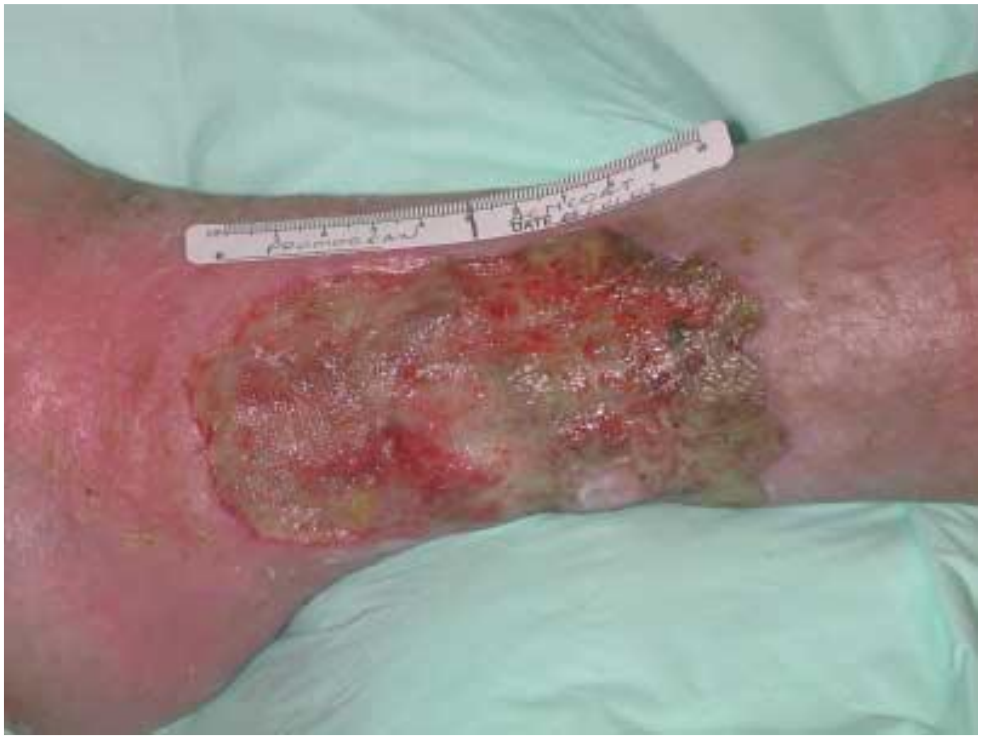

Figure 1: Chronic ulcer of the leg trapped in the inflammatory phase (Schultz *et al*.).[10]

1.2. Phases of wound healing

The wound healing process is arbitrarily divided into four highly integrated and overlapping phases: (i) haemostasis; (ii) inflammation; (iii) proliferation and (iv) tissue

remodelling (Figure 2).[11] These phases and physiological processes must occur in the proper sequence, at a specific time, and continue for a specific duration at an optimal intensity.[12, 13] The duration of this cascade of events differs for acute and chronic wounds. Wounds that exhibit impaired healing, such as delayed acute wounds and chronic wounds, generally, have failed to go through the normal stages of the complex healing process.[1, 13] However, it is important to note that there are no defined boundaries between the wound healing stages, as they overlap.[11]

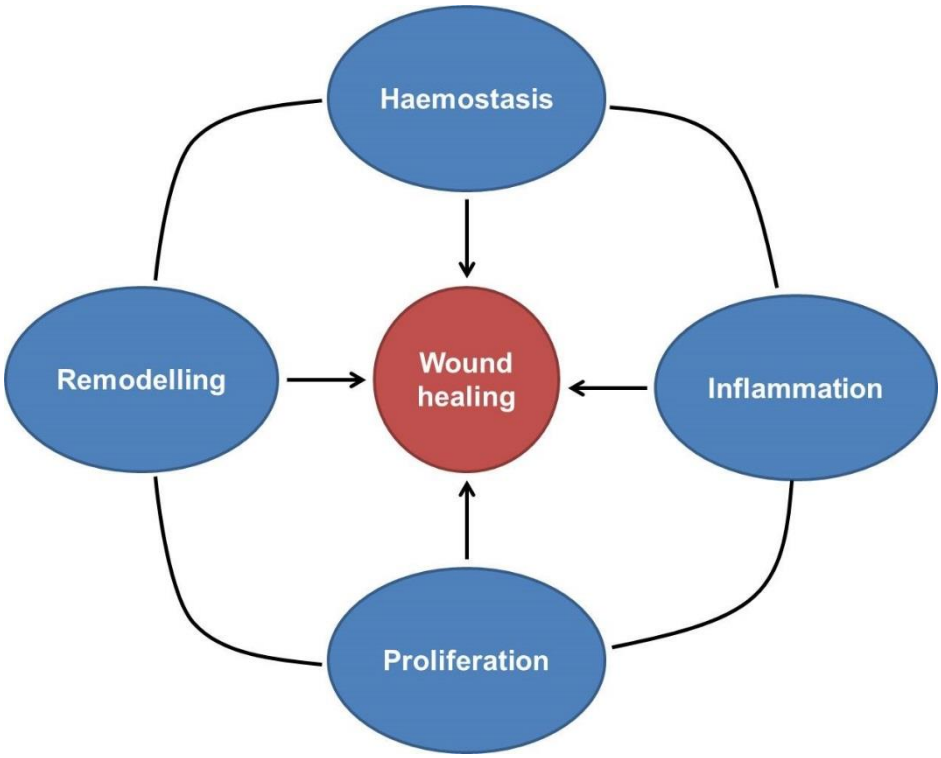

Figure 2: Phases of the wound healing process.

1.2.1. Haemostasis

Haemostasis starts immediately after wounding (Figure 3), when tissue is disrupted and blood vessels are severed, resulting in the release of blood into the wound site.[14] Haemostasis consists of the initial vascular constriction and fibrin clot formation which acts as a temporal barrier that prevents excess bleeding and limits the spread of pathogens into the blood system.[14] This is a way to protect the vascular system, keeping it intact, so that the function of the vital organs is not negatively affected due to the injury.[13]

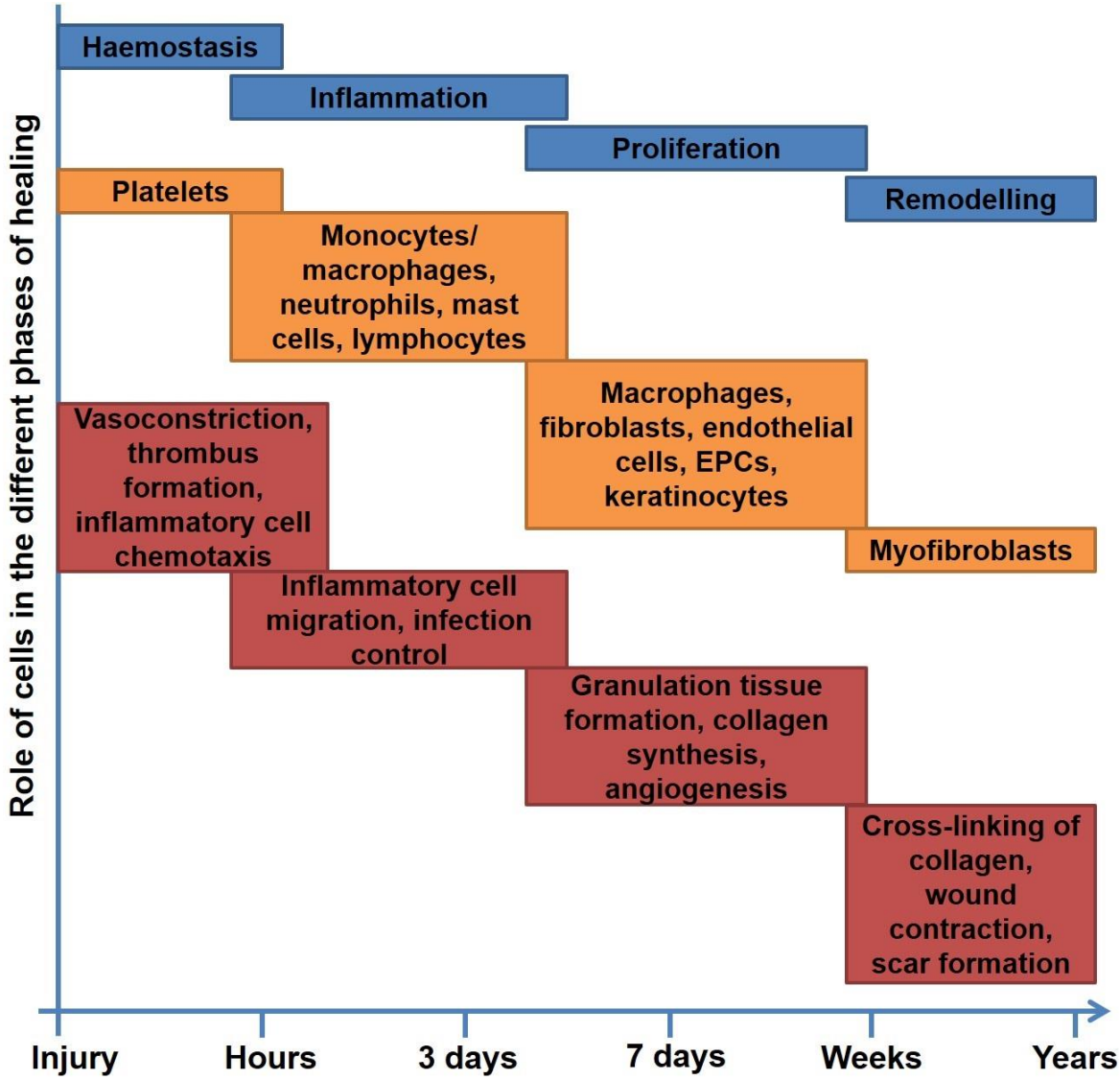

Figure 3: The wound healing process (Baltzis *et al*., 2014).[11] EPCs, endothelial progenitor cells.

Platelets play a major role at this stage of the wound healing process. Exposure of circulating platelets to collagen of the injured tissue leads to their activation, aggregation and adhesion to the damaged endothelium.[13] As platelet aggregation proceeds, platelet activation mediators, such as adenosine diphosphate (ADP) and thromboxane A2, are released. Following activation, platelets produce thrombin which initiates the coagulation cascade, resulting in a fibrin clot formation at the wound site (Figure 4).[14-16] The fibrin clot serves as a provisional matrix that acts as a scaffold for cells at the wound site, and contributes to the eventual formation of granulation tissue. Platelets also release cytokines or growth factors, such as platelet-derived growth

factor (PDGF) and transforming growth factor-β (TGF-β) that promote wound healing by initiating the chemotaxis of neutrophils and monocytes.[1, 13, 17]

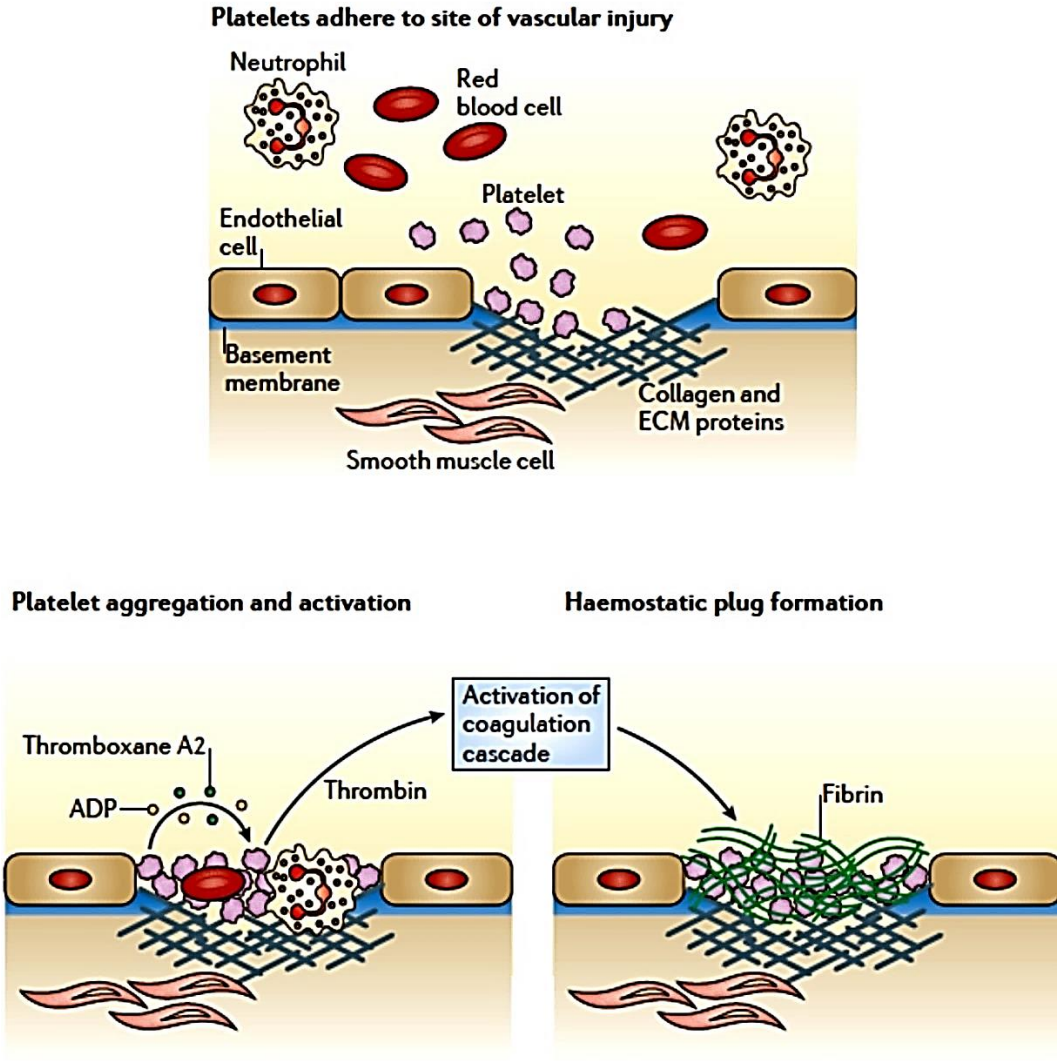

Figure 4: Fibrin clot formation (Semple *et al*.).[16] ADP, adenosine diphosphate; ECM, extracellular matrix.

1.2.2. Inflammatory phase

Once bleeding is controlled, the humoral and cellular inflammatory phase follows, with the aim of establishing an immune barrier against invading micro-organisms (Figure 3).[14] After the clot is formed, a cellular distress signal is sent out leading to infiltration of the wound vicinity by inflammatory mediators such as macrophages, neutrophils and mast cells (Figure 5). Neutrophils are the first cells to be drawn into the wound site by the activity of chemotactic cells or growth factors, such as interleukin (IL)-1, tumour necrosis factor (TNF)-α, TGF-β and platelet factor 4 (PF-4).[13]

Neutrophils promote wound healing by their involvement in infection control, tissue debridement, production of several growth factors that promote proliferation of cells, and proteases that degrade extracellular matrix.[11] They ensure an infection-free wound environment by ingesting and destroying foreign material, such as bacteria, by phagocytosis. The bacteria are destroyed within the phagosome by the action of the proteolytic enzymes, myeloperoxidase and lysozyme, and by an oxygen-dependent respiratory burst that produces reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet}$) and other oxygen (O_2) free radicals.[1, 11] Through this phagocytic action, bacteria, foreign particles and damaged tissue are removed, paving the way for subsequent processes.[1, 11]

Other cells which play important roles at this stage of the healing process are monocytes (macrophages) and resident mast cells. Macrophages aid in the removal of foreign bodies, dead cells and damaged tissue components, as well as the expression of various pro-inflammatory mediators and cytokines.[17, 18] These cells are crucial in wound healing to the extent that their depletion causes serious healing disturbances.[18] On the other hand, mast cell degranulation releases cytokines and proteases that, respectively, induce neutrophil recruitment and degrade the extracellular matrix, facilitating the healing process.[19] T-lymphocytes, which seem to have modulatory activity on tissue remodelling, enter the wound site in the late phase of inflammation.[11, 20]

1.2.3. Proliferative phase

After haemostasis has been achieved and an immune response has been triggered, the wound shifts towards tissue repair or proliferation (Figure 3). Characterized by fibroblast migration and deposition of newly synthesized extracellular matrix, this phase starts on the third day after wounding and lasts for about two weeks thereafter.[11]

Macrophages present in the wound release growth factors, including PDGF, vascular endothelial growth factor (VEGF), TNF-α and fibroblast growth factor (FGF), which act as chemoattractants for fibroblasts.[17] After migration into the wound area, fibroblasts populate the wound within a few days after injury (Figure 5), where they form a new

loose extracellular matrix that comprises of collagens, elastin, glycoproteins, proteoglycans, and glycosaminoglycans.[13]

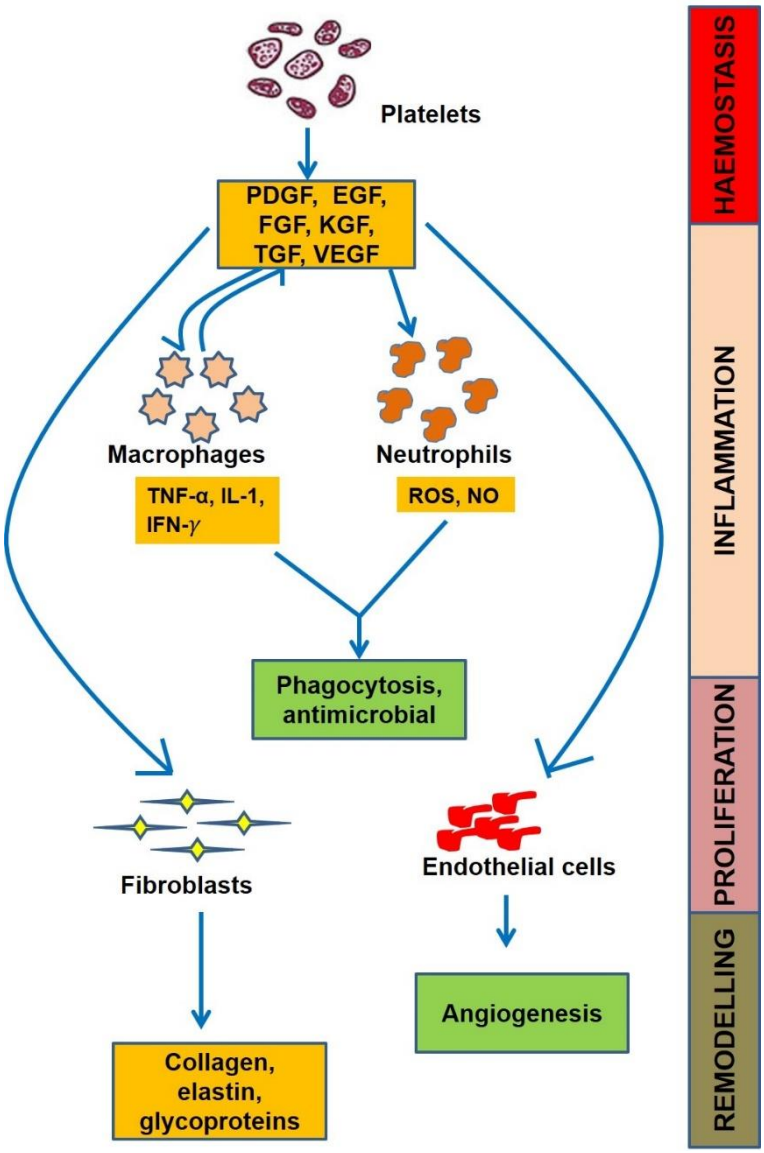

Figure 5: Cellular events in the wound healing process. PDGF, platelet-derived growth factors; EGF, epidermal growth factor; FGF, fibroblast growth factor; KGF, keratinocyte growth factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; TNF, tumour necrosis factor; IL, interleukin; INF, interferon; ROS, reactive oxygen species; NO, nitric oxide.

Collagen synthesis is an integral part of granulation tissue formation, imparting integrity and strength to all tissues. Furthermore, it forms the foundation for the formation of intracellular matrix within the wound, paving the way for other wound

healing processes, including wound contraction, regeneration of blood vessels and keratinocyte movement which result in wound closure.[1, 11]

1.2.4. Remodelling phase

The remodelling phase is responsible for the development of new epithelium and maturation of granulation tissue into scar tissue (Figure 3). This phase is tightly controlled by regulatory mechanisms with the aim of maintaining a delicate balance between degradation and synthesis of tissue components, leading to normal healing.[11] Several cells, such as myoblasts, contribute to this process through the release and deposition of synthesized collagen.[11, 17] Along with intracellular matrix maturation, collagen bundles increase in diameter, and hyaluronic acid and fibronectin are degraded.[11, 17] The tensile strength of the wound increases progressively in parallel with collagen collection, with the end result being a fully matured scar with a decreased number of cells and blood vessels, as well as a high tensile strength.[11, 17]

1.3. Barriers to wound healing

Non-healing wounds are a major global problem as almost every country is challenged with increasing expenditure on resources for wound care.[21] The increasing number of patients with chronic wounds is particularly due to the rise in diabetic foot ulcers, leg ulcers and pressure ulcers among the aged population and other susceptible patients.[20, 21]

Acute wounds which go through the phases of wound healing in a sequential and timeous manner, lead to the resolution of a wound within a few weeks. Chronic wounds, however, fail to follow this sequence of events uninterruptedly, and therefore take a much longer time to heal.[13] Reasons for this delayed healing are many and various.

Generally, wound healing depends on a multiplicity of factors, including patient age, wound size, depth and location, and the presence of local or systemic disease.[14, 22] Notwithstanding this general perspective, however, acute wounds usually show low levels of bacteria, inflammatory cytokines, proteases and ROS.[13, 22] Non-healing wounds on the other hand are associated with reduced oxygenation, chronic inflammation, fibroblast senescence, impaired function and levels of critical cytokines,

growth factors and their receptors, abnormal matrix metalloprotease (MMP) activity and infection (Figure 6).[13, 22]

An unregulated production and activity of proteases, including MMP, has been observed in chronic wounds.[23, 24] Examination of fluid from patients with chronic wounds shows a significant increase in the concentration of various metalloproteases and serine proteases[24]. Elevated levels of these proteases has been associated with degradation of fibronectin and some growth factors.[22, 24] This impedes the successful remodelling of the extracellular matrix since fibronectin is an essential protein involved in this process.[23, 24]

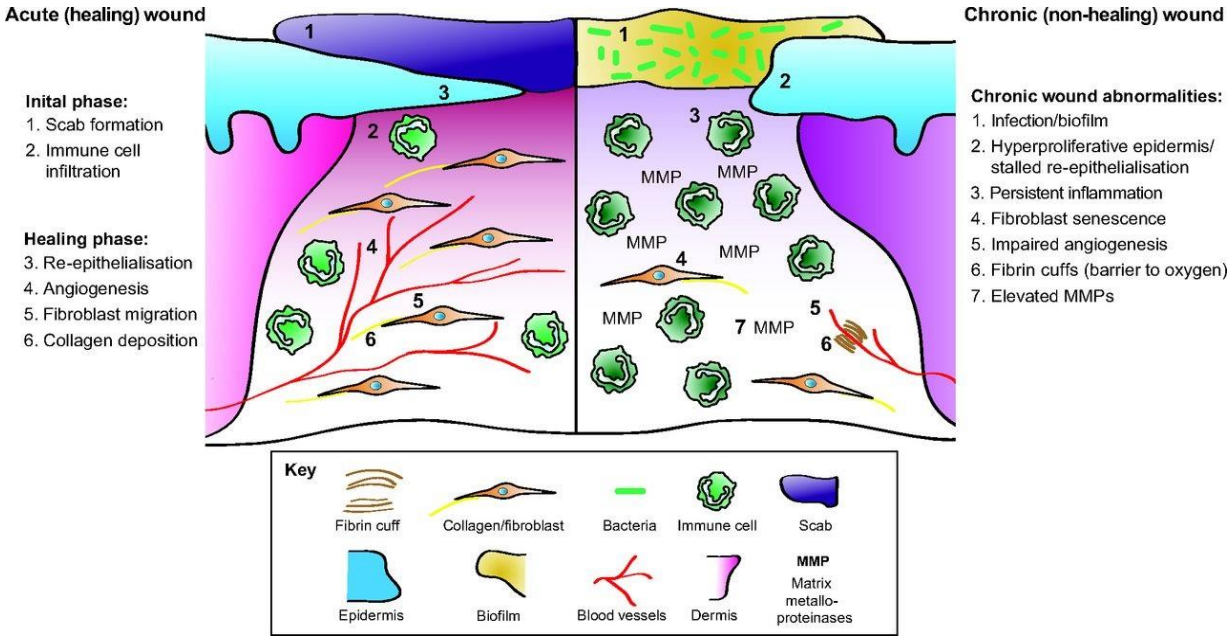

Figure 6: Biomolecular differences between healing and non-healing wounds.[23] MMP, matrix metalloproteinase.

1.3.1. Oxidative stress

Reactive oxygen species, such as H_2O_2, O_2• and hydroxyl radical (OH•), generated as by-products in the inflammatory phase of the wound healing process could be deleterious to wound healing due to their harmful effects on cells and tissues.[25] Though ROS play positive roles in pathogen destruction and angiogenesis, high levels could cause damage to lipids, proteins and deoxyribonucleic acid (DNA).[26] This oxidative stress caused by ROS could also lead to persistent inflammatory cell

infiltration during the wound healing process, hence impairing healing and allowing for chronic wound development.[25, 26]

For protection against ROS, human cells generate enzymes, such as superoxide dismutase (SOD) and catalase, as well as antioxidant compounds like reduced glutathione (GSH).[27] These antioxidant enzymes and compounds suppress free radicals and ROS, and thus prevent their build up to toxic proportions (Figure 7). Therefore, administration of exogenous substances with antioxidant properties, such as ascorbic acid, could be a good therapeutic strategy for the prevention of free radical and ROS-induced disease conditions.[28]

The generation and metabolism of ROS is a complex procedure involving several enzyme systems.[24] Superoxide, a common ROS produced by the activities of reduced nicotinamide dinucleotide phosphate (NADPH) oxidase, xanthine oxidase (XO), nitric oxide synthase (NOS), lipoxygenase, and mitochondrial enzymes, is a major source of several other ROS. Superoxide is converted by SOD to H_2O_2, which, in turn, is reduced to water by catalase, glutathione peroxidases (GPx), and peroxiredoxins (Prx). The latter two enzymes catalyse a reaction that causes oxidation of thioredoxin and GSH in the process. In the presence of reduced transition metal (Fe^{2+}, Cu^{+}), H_2O_2 can undergo spontaneous conversion to OH•, or related metal associated reactive species, which is extremely reactive. Importantly, nitric oxide (NO) can be rapidly inactivated by reaction with O_2• which leads to the production of the strong oxidant, peroxynitrite ($ONOO^{-}$). For this reason, SOD is a first line of defence against the toxicity of superoxide anion radicals. The enzyme also participates in cell signalling via regulating ROS (e.g., O_2•, H_2O_2) and available NO.[27, 28]

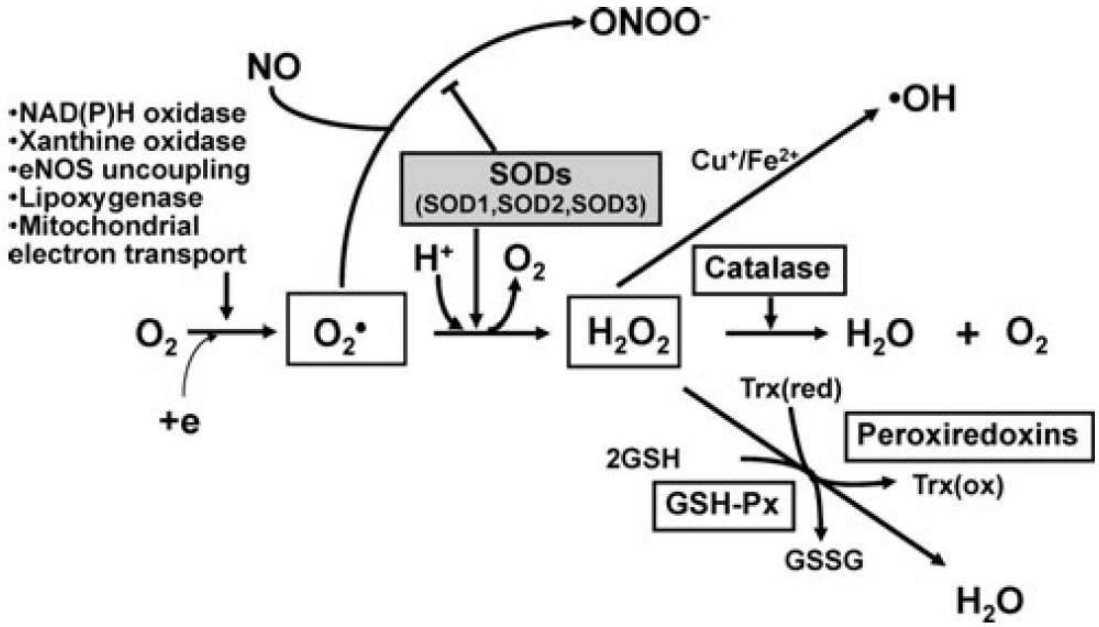

Figure 7: Generation and metabolism of reactive oxygen species.[27] O_2, oxygen; O_2•, superoxide; NADPH, reduced nicotinamide dinucleotide phosphate; NOS, nitric oxide synthase; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; H_2O, water; GSH, reduced glutathione; GSH-Px, glutathione peroxidases; GSSG, oxidised glutathione; Prx, peroxiredoxins; Fe^{2+}, reduced iron; Cu^{+}, reduced copper; OH•, hydroxyl radical; NO, nitric oxide; NOS, nitric oxide synthase; $ONOO^{-}$, peroxynitrite; Trx(red), reduced thioredoxin; Trx(ox), oxidised thioredoxin.

1.3.2. Microbial infection

The wound forms a fertile ground for growth of microorganisms such as bacteria. Several microbial organisms, including *Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* have been isolated from various wounds.[29] These microorganisms hinder wound healing by mainly prolonging the inflammatory process.[5]

Inflammation, which is important for the removal of these microorganisms, may be prolonged in the absence of effective decontamination, leading to prolonged elevation of pro-inflammatory cytokines like IL-1 and TNF-α.[5] This continuous influx of pro-inflammatory cytokines leads to the increased release of cytotoxic enzymes, ROS and

MMPs that could provoke extensive tissue damage, and cause prolonged healing of the wound.[5, 30]

1.3.3. Inflammatory response

As stated earlier, the inflammatory response starts immediately after injury has occurred, with the infiltration of immune cells into the injured area.[13] A normal inflammatory response is an acute response that resolves after removal of the inciting stimulus.[22] However, when the normal inflammatory response progresses to a chronic response because of long-term inappropriate responses to a stimuli or the inability to remove offending agents, tissue damage or disease can occur.[20, 22] Apart from the potential of chronic inflammation to promote extended wound healing, usually due to chronic infections, it is also responsible for a number of inflammatory conditions such as rheumatoid arthritis, asthma and inflammatory bowel disease.[11]

Chronic wounds which are in the process of healing have decreased levels of cytokines such as TNF-α, interleukin 1β, and TGF, compared to non-healing chronic wounds (Figure 6).[11] This suggests a correlation between non-healing wounds and increased levels of pro-inflammatory cytokines, thus significantly altering the normal inflammatory process in this group of patients. Some other studies link chronic wounds with suppressed macrophage activation and altered lymphocyte infiltration.[18] Hence the release of cytokines and growth factors for the recruitment of fibroblasts, keratinocytes and endothelial cells would be impaired in patients with chronic wounds, thus contributing to the arrest of healing in the inflammatory phase.[18, 22, 23]

1.4. Treatment of wounds

Many treatment options, both pharmacological and non-pharmacological, are available for wounds. Non-pharmacological modalities such as debridement, moisture-retentive dressings, negative pressure therapy, hyperbaric oxygen therapy and biologic skin substitutes, including epidermal, dermal and dermo-epidermal combination constructs, have proved useful for decreasing wound healing time, especially in the absence of infection and excessive inflammation.[31] However, each one of these treatment options is bedevilled with various traits that limit its use among a great number of people across the world. While a lot of them are simply too expensive for most people to afford, others, such as surgical debridement, are not

suitable for certain wound types, including ischemic limbs and heel ulcers that are close to the bone.[32]

1.4.1. Pharmacologic treatment of wounds

Because of the significant roles played by bacterial infection and excessive inflammation in derailing the wound healing process,[5, 30] pharmacological treatment is geared towards the prevention and treatment of infection, as well as management of inflammation.[10] Treatment of microbial infections involves the use of a host of antimicrobial agents either topically, systemically, or both. However, treatment is fraught with antimicrobial resistance, as several antibiotic-resistant bacteria, such as the methicillin-resistant *Staphylococcus aureus* (MRSA), glycopeptide-resistant *Enterococci*, and multidrug-resistant strains of *Acinetobacter baumanii*, and *Pseudomonas aeruginosa,* emerge as a result of inappropriate use of antibiotics.[29] To further worsen the plight of antibiotics, some bacteria form a protected microenvironment known as biofilms.[5] This is a complex community of aggregated bacteria embedded in a self-secreted extracellular polysaccharide (exopolysaccharide) matrix which makes them more resistant to conventional antibiotic treatment.[33] Drug-resistant bacterial strains and biofilms have the potential of further extending the wound healing time.[23, 31, 33]

Microorganisms use a series of mechanisms to overcome the effect of antibiotics (Figure 8). These include prevention of access to drug targets, changes in structure or protection of antibiotic targets, and direct modification or inactivation of antibiotics.[34] For example, as shown in Figure 8, β-lactam antibiotics target the penicillin-binding protein (PBP). Antibiotic A can enter the cell via a membrane-spanning porin protein, reach its target and inhibit peptidoglycan synthesis. Antibiotic B can also enter the cell via a porin, but unlike Antibiotic A, it is efficiently removed by efflux. Antibiotic C cannot cross the outer membrane and so is unable to access the target PBP.[34] As a result of this, several antibiotic combinations are required for effective treatment of wound infections (Table 1), hence further escalating the already expensive cost of treatment.[10]

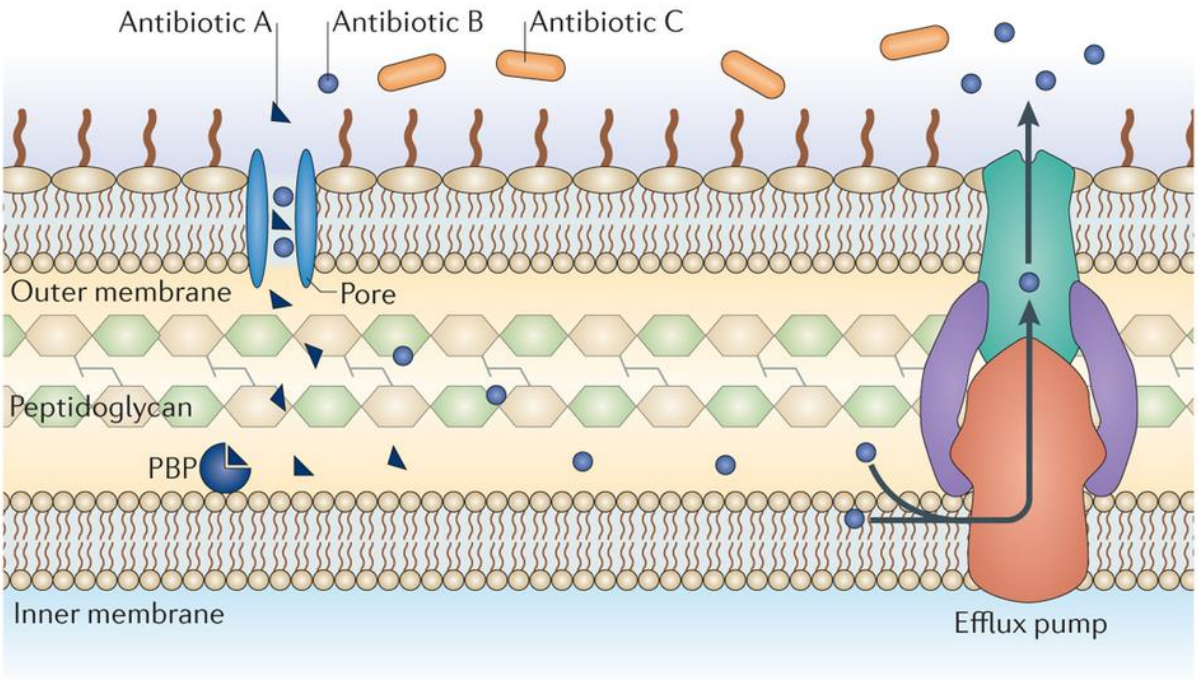

Figure 8: Antibiotic resistance mechanisms. (Reproduced with permission)[34] PBP, penicillin binding protein.

Table 1: Antimicrobial therapy for chronic wounds.[10]

Presentation	Severity	Organisms	Antibiotic/dose	Route	Duration
• Wound < 4 weeks old • <2 cm rim of cellulitis • Superficial infection • No systemic response • No bone involvement • Outpatient management	Mild	• *S. aureus* • *Streptococcus spp*	• Cephalexin 500 mg qid, or • Clindamycin 300 mg tid	PO	14 days
• Wound <4 weeks old • Superficial infection • Extensive cellulitis • Systemic response • Inpatient management	Severe	• *S. aureus* • *Streptococcus spp*	• Cloxacillin, or • Oxacillin 2 g q6h	IV (step down to PO)	14 days

Presentation	Severity	Organisms	Antibiotic/dose	Route	Duration
• Wound >4 weeks old • Deep tissue infection • No systemic infection • Outpatient management	Mild to moderate	• *S. aureus* • *Streptococcus spp* • *Coliforms* • *Anaerobes*	• Amoxicillin-clavulanate 500/125 mg tid, or • Cephalexin 500 mg qid + metronidazole 500mg bid, or • Cotrimoxazole 160/800 mg bid + metronidazole (or clindamycin), or • Clindamycin 300 mg po tid + levofloxacin 500 mg po od	PO	2 to 12 weeks
• Wound >4 weeks old • Deep tissue infection • Systemic response with fever, rigors • Limb or life threatening • Inpatient management	Severe	• *S. aureus* • *Streptococcus spp* • *Coliforms* • *Anaerobes* • *Pseudomonas*	• Clindamycin 600 mg q8h + cefotaxime 1g q8h (or ceftriaxame 1gm q24h), or • Piperacillin 3g q6h + gentamicin 5mg/kg q24h, or • Piperacillin-tazobactam 4.5g q8h, or • Clindamycin 600mg q8h + levofloxacin 500mg q24h, or • Imipenem 500mg q6h	IV	14 days IV (prolonged oral therapy if bone or joint involvement)

PO, per os; q, every; IV, intravenous; bid, two times a day; tid, three times a day; qid, four times a day.

The use of topical agents, such as products containing low iodine concentrations, and silver preparations such as silver sulfadiazine, have shown effectiveness against wound pathogens, thereby enhancing healing.[35, 36] Other antiseptic agents such as sodium hypochlorite solution, H_2O_2, mercuric chloride, crystal violet, chlorhexidine and acetic acid are similarly useful topical agents for preventing infection in wounds.[37]

Systemic antibiotics, often reserved for increased bacterial burden of the superficial compartment that does not respond to topical treatment, are also available for wound treatment. The list includes cell wall inhibitors like the penicillins and cephalosporins; protein synthesis inhibitors such as the tetracyclines and lincosamides, among others.[31]

Though the introduction of antibiotic therapy into wound treatment has resulted in tremendous improvement in the healing rate, their use has been limited by antimicrobial resistance. This has rendered a number of antibiotics such as the β-lactams, fluoroquinolones and aminoglycosides ineffective at treating microbial infections.[34] Apart from this, antibiotic use is associated with diverse adverse effects. Topical antibiotics such as gentamycin and neomycin frequently cause contact dermatitis in chronic wounds, while commonly used iodine containing products such as povidone iodine have been shown to be cytotoxic.[31]

1.4.2. Ethnomedicines

The use of herbs and other natural products for the restoration of health to patients suffering from disease conditions has a long history, with virtually all cultures across the globe either using or having a history of medicinal plant use as a source of medicine.[38] Herbal medicine is still the main form of treatment, especially among indigenous populations around the world and those living in under developed countries.[39] According to a 2013 report by the WHO, the majority of the world's population still depend on complementary and alternative medicine as a source of medication.[38] In Ghana, like most parts of the world, orthodox and alternative medicines operate side by side[38] in the provision of health care for the citizenry. Currently, over 65% of Ghanaians rely on providers of herbal medicine for primary healthcare.[39]

Apart from accessibility, people use herbal products for diverse reasons. For a large proportion of individuals, the cost of orthodox medicine is often quite expensive, hence traditional medicine remains the only alternative.[38] Additionally, the recent proliferation of media outlets that promote the use of all forms of herbal medicine seem to influence its use. Unlike conventional therapies that are regulated by law, herbals are often seen as complementary medicine not requiring regulation. However, a lot of

countries now implement regulations that are aimed at protecting consumer health by ensuring that herbal medicines are safe and of high quality.[38] In South Africa, the South African Health Products Regulatory Authority (SAHPRA) regulates the use of all medicines and medicine-related substances such as herbs.[40] Recently the European Union (EU) implemented a directive to harmonize the regulation of traditional herbal medicine products across the EU and establish a simplified licensing system in order to help the public make informed choices about the use of herbal products.[41]

The importance of traditional medicine even extends into conventional or modern medicine. Several clinically approved medications have either been derived directly or indirectly from medicinal plants.[42] The list includes therapeutic agents such as the opioid analgesics (morphine, codeine and methadone) from *Papaver somniferum*, the local anaesthetic agent; cocaine, from *Erythroxylum coca*; the non-steroidal anti-inflammatory drug, aspirin from *Salix alba*; the cardiac glycoside, digitoxin, from *Digitalis purpura*; the antimalarial drugs quinine and artemisinin from *Cinchona sp.* and *Artemisia annua*, respectively; and the chemotherapeutic agent, paclitaxel, from *Taxus brevifolia*.[42] The list of drugs derived from plants underscores the critical role of ethnomedicinal practice and research in modern medicine.

Some phytochemical constituents are known to promote wound healing through their antimicrobial activity, antioxidant and radical scavenging activity, or by enhancing various processes within the phases of wound healing such as haemostasis, proliferation and remodelling.[43] Curcumin, a polyphenolic compound from *Curcuma longa*, enhanced wound healing by quenching free radicals, reducing inflammation, improving collagen deposition, and facilitating angiogenesis.[43, 44] Also, arnebin-1, a naphthaquinone derivative from *Arnebia nobilis*, reduces wound healing time through its antimicrobial activity and ability to increase re-epithelialization.[43] A topical application of 0.4% solution of asiaticoside, a compound isolated from *Centella asiatica*, facilitated healing of wounds in diabetic rodents by increasing wound tensile strength and epithelialization.[45] The ability of chlorogenic acid, a polyphenolic compound present in plants, to promote wound healing has also been reported.[46] This has been attributed to the compound's antioxidant and anti-inflammatory effects, although it could exhibit prooxidant effects upon prolonged treatment.[46] Other

polyphenolic compounds like quercetin and gallic acid are also known to facilitate wound healing.[47, 48] However, although plants are known to possess these active phytochemicals with potential for use in wound healing, not many have been isolated. [12]

1.5. Models for assessing wound healing activity

Several models have been used to measure the efficacy of various wound healing compounds in a scientific manner. These models have been classified into *in vivo* and *in vitro* methods which are discussed below.

1.5.1. *In vivo* models

In vivo models, which usually involve the use of small rodents such as rats, are essential for examining various stimuli in a complex environment consisting of all the cells involved in wound healing as discussed above (Figure 3).[11] These models include the granuloma, incision, open-wound, and burn models which are designed to test the ability of an animal to induce an inflammatory response, test tensile strength of healing incisions, examine rate of wound closure, and determine systemic response to thermal injury, respectively.[49]

Although *in vivo* rodent models are advantageous because the animals are small, easy to handle, and relatively cheap, high degree of phenotypic variability in skin exists between rodents and humans. Therefore, an accurate model of human wound healing should use an animal with similar skin characteristics. Rodent models have several limitations, including a predominant contractile wound-healing phenotype due in part to the well-developed panniculus carnosus layer of striated muscle that contracts more than human skin,[50, 51] and a much higher epidermal appendage density with hair follicles that differ from those of humans.[52] Furthermore, the murine epidermis is only 50 μm thick, so it is technically difficult to create partial-thickness wounds.[52]

Porcine wound models have thus emerged as promising alternatives for studying wounds. Pig skin has a lot more similarities with humans compared to rodents. Like humans, they have a relatively thick epidermis, distinct rete pegs, dermal papillae, and dense elastic fibers in the dermis.[53, 54] The histologic location of dermal collagen IV, fibronectin, and vimentin, is also similar.[55] Pigs also have sparse hair rather than fur,

although the hair is coarser than human hair.[54] Unlike rodent, rabbit, and canine skin, which is loose and slides over the subcutaneous fascia, porcine skin is adherent to the underlying structures, similar to human skin.[52] Moreover, the immune cells in pig skin, including dendritic cells, are the same as those in human skin.[56] Therefore, numerous pig models have been designed to study the wound healing process. These include models for chronic ischemic/non-ischemic wounds, diabetic wounds, burns, hypertrophic scars, and wound infections.[57]

However, the use of animal models is severely curtailed in many parts of the world for a variety of reasons. These include the rising ethical concerns about the use of animals in research, high cost of animal studies, and the unsuitability of the models for certain types of research, such as the bioassay-guided fractionation of compounds responsible for biological activity.[58]

1.5.2. *In vitro* models

Generally *in vitro* models of wound healing are simple, rapid and involve minimal ethical consideration compared to whole animal experiments. Furthermore, they allow insight into the biochemical and physiological processes induced by the test agent. In spite of the widespread use of *in vitro* models, they are still inferior to *in vivo* or whole animal studies because no single *in vitro* assay comprises all the complexities of a disease state.[49]

In these models, various mechanistic questions related to tissue repair are answered by assessing the effect of chemical compounds on cultured cells, tissues and organs. This is achieved by examining one of the key components of tissue repair: inflammation, proliferation, scar formation, angiogenesis, re-epithelialization, and contraction.[1] Additionally, because wound healing can be impaired by factors such as ROS and microbial infections, exogenous factors are also examined in *in vitro* experimentations.[27]

Several chemical substances have been investigated using various techniques to assess their ability to impair eicosanoid synthesis, and by so doing suppress inflammation. Most studies investigated the effect of compounds on the two enzymes, cyclooxygenase and 5-lipoxygenase, responsible for the conversion of arachidonic

acid to prostaglandins and leukotrienes.[59] In this model, various prostaglandins and leukotrienes are measured by radioimmunoassay techniques at specific times after the enzyme-substrate reaction.

Proliferation of cultured fibroblasts and other cells involved in wound healing can be assessed by determination of total protein of viable cells by staining with an appropriate dye such as sulforhodamine B (SRB) or by metabolism of a coloured substance such as 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT). [58]

To understand the effect of compounds on scar formation, models examine key components involved in scar formation, such as production of extracellular matrix (ECM) by fibroblasts and other cells. In an effort to determine mechanisms responsible for keloid formation, a microassay is available to measure levels of collagenase-digestible radiolabelled proteins.[60] Several other studies have demonstrated how fibroblast cells isolated from various tissue types influence scar formation using similar *in vitro* techniques.[61-63]

When placed in three-dimensional matrices, isolated endothelial cells develop tubular structures that resemble capillaries. [64] This model, used to assess angiogenic potential of substances, consists of a disc into which the test substance is deposited, and subcutaneously implanted into a host animal and examined periodically. The chorioallantoic membrane of chicken egg has also been used to test the angiogenic potential of different agents by injection into the membrane and documenting development of new vessel growth.[65, 66]

For examination of re-epithelialization, the rate of migration of isolated keratinocytes has been studied by various techniques which involve the creation of a gap or “wound” between confluent cells and the time until closure determined as rate of re-epithelialization.[67] Addition of different types of ECM and the use of time-lapse video microscopy has shown that keratinocyte migration is determined by which stimuli it encounters.[68]

The fibroblast-populated collagen matrix has been used to assess the effect of chemicals on wound contraction.[69] In this model, fibroblasts are placed into a disc-shaped collagen matrix which contracts over time as the cells contract and migrate. All these *in vitro* models discussed involve manipulations within single cell cultures. However, various techniques have involved combined cells, tissues or organ cultures. The co-culturing of fibroblasts and keratinocytes, for instance, has been shown to augment production of growth factors.[70] Moreover, composite skin and other organs have also been used to demonstrate how heat stress affect cells.[71, 72]

In the current study, various *in vitro* techniques were used to assess three Ghanaian plants for biological activity related to wounds. These models have been chosen mainly on the basis of their effectiveness, widespread use, and suitability for the study.

1.6. Ghanaian medicinal plants selected for the study

Herbal medicine remains the most accessible form of treatment for diverse medical conditions including wounds. In spite of their long use, most of the medicinal plants have not been evaluated scientifically. Particularly worrying is the paucity of information on traditional wound healing remedies and their potential as source of lead compounds for synthesis of drugs for wound treatment. Though some researchers have attempted to bridge this gap,[12, 73] a lot remains to be done.

Three medicinal plants traditionally used for treating wounds are *Boerhavia diffusa* Linn, *Aspilia africana* (Pers.) C.D. Adams and *Erythrina senegalensis* DC. In this study, the above-mentioned medicinal plants were chosen because of the paucity of evidence in the scientific literature regarding their wound healing potential. Although the mode of ethnomedicinal usage was considered in selecting plant parts, other factors were considered. For instance, *A. africana* leaves and flowers are traditionally used for managing wounds.[74, 75] However, studies involving whole plant extracts of the plant indicate presence of various phytochemical components, such as flavonoids and phenols, with relevance to wound healing.[76] As a result, whole plants of *A. africana* were used in the current study. Also, whole plants of *B. diffusa*,[77, 78] and leaves of *E. senegalensis*,[79] were used in accordance with the traditional practice since no data was sighted to suggest that other parts may be advantageous in the management of wounds.

1.6.1. Ethnomedicinal relevance of *Aspilia africana*

Aspilia africana (Pers.) C.D. Adams (Figure 9) is a herb belonging to the family Asteraceae, and commonly found in old farmlands and open places in the forests of tropical West Africa.[80] It is commonly known as “Mfufu” in the Central region of Ghana (Fante). Due to its ability to stop bleeding from fresh wounds, it is widely referred to as the haemorrhage plant. The crushed leaves have been used to clean surfaces of sores in order to promote healing.[75] A decoction of the leaves is also used in pulmonary hemorrhages,[75] and for the treatment of malaria symptoms in East and Central Africa.[80]

Figure 9: Photomicrograph of aerial parts of *Aspilia africana.*

1.6.2. Ethnomedicinal relevance of *Boerhavia diffusa*

Boerhavia diffusa Linn (Figure 10) is a member of the Nyctaginaceae family. It is a perennial creeping or climbing herbaceous plant also known as “Nkokodwe” (Akan, Ghana) or pig weed. Widely distributed in tropical and subtropical regions of the world, this plant is revered for its numerous ethnobotanical uses including wounds, infections and pain management.[81]

Figure 10: Photomicrograph of *Boerhavia diffusa.*

1.6.3. Ethnomedicinal relevance of *Erythrina senegalensis*

Erythrina senegalensis DC (Papilionaceae) is a thorny shrub or small tree with bright red flowers commonly grown in West Africa as an ornamental plant and one of the oldest known African medicinal plants.[82] It is commonly known as “Sorowa” (Akan, Ghana), the coral flower or parrot tree. *E. senegalensis* (Figure 11) is used in traditional medicine for the management of amenorrhoea, dysmenorrhoea, malaria, infection, wounds and body pains (chest pain, back pain, abdominal pain), headaches and body weakness, gastric ulcer, diarrhoea and constipation.[83] The aqueous extract of the bark is used for the treatment of jaundice in northern Nigeria, and an infusion mixed with lime and pepper is administered to cure venereal diseases.[75] The powdered bark and leaves are used in the form of soup to treat female infertility. The root infusion is used in Nigeria as a toothache remedy and in Ivory Coast for general disease treatment.[75]

Figure 11: Photomicrograph of aerial parts of *Erythrina senegalensis*.

1.7. Aim of the study

The aim of this study was to characterise the biological activities of the three selected medicinal plants from Ghana used in wound treatment.

1.8. Objectives

The objectives of this study were to:

- Prepare extracts from *A. africana, B. diffusa* and *E. senegalensis* sequentially using hexane, ethyl acetate, methanol and water, as well as an extract mimicking an ethnomedicinal preparation.
- Determine the phytochemical composition of the extracts using thin layer chromatography (TLC).
- Obtain chromatographic fingerprints from extracts and fractions using ultraperformance liquid chromatography, in tandem with time-of-flight mass spectrometry (UPLC-qTOF-MS).
- Evaluate extracts for possible cytotoxicity using the SRB assay, phase contrast, modified interference contrast (PlasDIC), and fluorescence microscopy.

- Determine the free radical scavenging activity of extracts using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH).
- To assess extracts for ability to attenuate AAPH-induced oxidative stress *in vitro*.
- Determine the anti-inflammatory activity of the extracts using the XO inhibition assay.
- Determine the effect of the extracts on cell migration using the scratch wound assay.
- Assess antimicrobial activity of the extracts against planktonic by using the disc diffusion and broth microdilution assays.
- To determine effect of extracts against biofilm forms of microorganisms using the crystal violet assay.

Chapter 2

Plant collection, preparation of extracts, and phytochemical analysis

2.1. Introduction

2.1.1. Usefulness of plants in healthcare

Humans have depended on nature for several decades for their basic needs, including curatives for a wide spectrum of diseases. In particular, plants have been useful in this respect. Several records of plant-derived substances used in ancient medicine exists. These include oils of *Cedrus species* (cedar) and *Cupressus sempevirens* (cypress), *Glycyrrhiza glabra* (licorice), *Commiphora species* (myrrh), and *Papaver somniferum* (poppy juice), which are used for treatment of coughs, infections, and inflammatory conditions.[84] Historical records from Egyptian medicine, Chinese Materia Medica, India, and the Greco-Roman world paint a picture of the significance of medicinal plants to the survival of the human race.[42, 84]

To date, plants continue to directly or indirectly serve the health needs of mankind. According to current statistics, a great majority of the world's population still directly rely on natural products for their health needs.[38] Furthermore, plants also play relevant roles in modern medical practice, serving as sources of lead molecules for development of several clinically used drugs for treatment of a broad range of diseases. The list of plant-derived clinical medications include the bronchodilating agent, sodium cromoglycate from *Ammi visnaga*; metformin and other bisguanidine-type antidiabetic drugs from *Galega officinalis*; and the antihypertensive agent, verapamil, from *Papaver somniferum*.[42] Other drugs, such as the antimalarial drugs quinine, chloroquine, mefloquine, artemisinin; antiasthmatic drugs such as salbutamol and salmeterol; the muscle relaxant, tubocurarine; and anticancer drugs such as vinblastine, vincristine, and paclitaxel, were all obtained either directly from medicinal plants or synthetically derived from plant compounds.[84] Several other plant-derived clinical drugs are playing major roles in modern medicine.

These examples attest to the relevance of medicinal plants as sources of useful therapeutic molecules for drug development. Therefore, research activities that are focused on evaluating the health benefits of medicinal plant extracts and plant-derived

compounds should be encouraged. Such activities could lead to identification of useful alternatives and novel therapeutics for disease treatment.

2.1.2. Methods for the extraction of phytochemical components

Extraction of active ingredients of plants into a suitable solvent or form is a pivotal aspect of the procedure to scientifically evaluate activity of traditional remedies, as well as the possible isolation of bioactive molecules (Figure 12). An extensive list of extraction methods has been employed to achieve this objective. These include maceration, infusion, decoction, percolation, and hot continuous extraction. More recent techniques, such as ultrasound-assisted solvent extraction, microwave-assisted solvent extraction, and supercritical fluid extraction, have also been described.[85] The nature and quality of an extracted component is dependent on the part of plant material, extraction solvent, and the extraction procedure employed (Table 2).

Active components can be derived from virtually all parts of the plant, such as the leaves, flowers, seeds, stem bark, and roots. However, these different parts may not necessarily possess the same phytochemical components even if they are derived from the same plant.[86] Some compounds inherent in *B. diffusa*, for instance, are only detected in the roots. This was noted in a study which was conducted to define the plant's qualitative and quantitative phenolic fingerprint for purposes of defining its chemical identity and for its quality control.[86] Also, samples obtained from different locations varied in phytochemical components. Other authors have come to the conclusion that secondary metabolite distribution may fluctuate between different parts of the same plant species.[87]

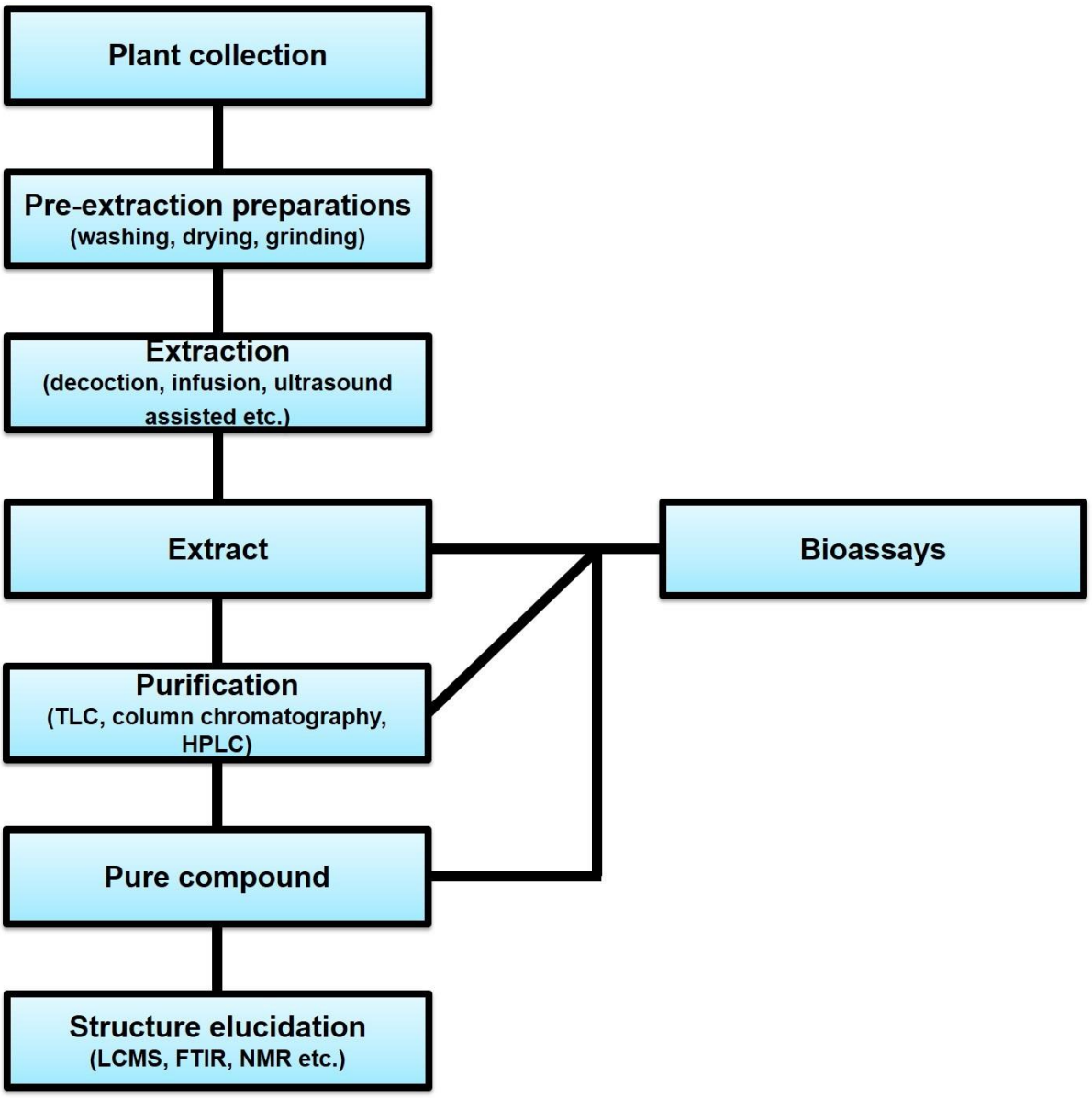

Figure 12: Schematic representation of events leading to bioactive component determination from plants. TLC, thin layer chromatography; HPLC, high performance liquid chromatography; LCMS, liquid chromatography-mass spectrometry; FTIR, Fourier-transform infrared spectroscopy; NMR, nuclear magnetic resonance

Table 2: A summary of methods used in plant extraction

Method	Description	Advantages	Disadvantages	References
Maceration	Soaking in solvent at RT for a minimum of three days with frequent agitation	• Simple • Cost effective	• Large volume of solvent used	Azwanida[88]
Percolation	Powdered plant material infused in a percolator and percolated at a slow rate (e.g. six drops/min)	• Simple • Cost effective	• Large volume of solvent used	Rathi *et al*.[89]
Infusion	Soaking in solvent at RT for a period less than three days with frequent agitation	• Simple • Cost effective	• Large volume of solvent used	Azwanida[88]
Decoction	Sample boiled in water for a defined period	• Simple • Cost effective	• Only suitable for extracting heat stable compounds • Large volume of solvent used	Azwanida[88]
Hot continuous (Soxhlet) extraction	Powdered plant material extracted with the aid of a Soxhlet apparatus by repeated vaporization and condensation of extracting solvent	• Minimum amount of solvent required	• Potential for environmental pollution • Limited to dry and finely divided samples • Compounds that are not heat stable may be destroyed	Amid *et al*.[90]
Ultrasound-assisted solvent extraction	Involves use of ultrasound ranging from 20 – 2000 kHz	• Simple • Cost effective • Reduced extraction time • Reduced solvent consumption	• Free radical formation	Kaufmann and Christen[91]
Microwave-assisted solvent extraction	Use of microwave energy to facilitate partitioning of analytes from the sample matrix into solvent	• Reduced extraction time • Minimal solvent volume	• Limited to compounds that are stable in microwave energy	Kaufmann and Christen[91]
Supercritical fluid extraction	Uses supercritical fluid such as CO_2	• Reduced extraction time	• High cost of equipment	Patil *et al*.[92]
Counter-current extraction	The material to be extracted is moved in one direction within a cylindrical extractor where it comes in contact with the extraction solvent	• Efficient and effective	• High cost of equipment	Handa *et al*.,[93]

RT, room temperature; CO_2, carbon dioxide

The phytochemicals extracted also depends on the form of the plant material. Extracts could either be obtained from the fresh or dried plant material. It has been reported that both drying and extraction conditions can significantly impact on the constituents and bioactivity of the extracts.[94] In their study, Yi and Wetzstein recounted that both drying and extraction conditions impacted total polyphenol (TPP) and Trolox equivalent antioxidant capacity (TEAC) of extracts.[94] The sun-dried and 40ºC oven-dried herbs exhibited significantly higher TPP content and antioxidant capacity, compared to 70ºC oven-dried samples.[94] Furthermore, drying samples at 70ºC caused significant antioxidant loss compared with low-temperature drying,[94] highlighting the importance of the starting plant material and preparation on the outcome of the extraction process. This suggests that low-temperature drying may not only be a good means to preserve medicinal/culinary herbs, but provides improved biochemical activity of thermolabile compounds.[94]

Also, samples extracted with 80% ethanol had significantly higher TPP and TEAC, compared with those extracted with 80% methanol.[94] Therefore, the choice of solvent used in extraction also determines the nature of biologically-active plant components extracted. A host of solvents are available for extraction purposes. These range from aqueous (water) to organic solvents, or a combination of both. A single medicinal plant is composed of a complex mixture of phytochemicals with varying polarities.[95] Therefore, the polarity of the extracting solvent largely determines which components are extracted, with non-polar components generally being extracted into non-polar solvents, and polar components into polar solvents. Not only did the extracting solvent influence the extraction yield of *Quercus coccifera* L. and *Juniperus phoenicea* L. fruit extracts, but also the TPP content and bioactivities.[95] This was identified in a study to investigate the effects of different extracting solvents on TPP and antioxidants, as well as antibacterial activity of the plants.[95] The choice of solvent is thus influenced by what is intended with the extract as well as the targeted compounds to be extracted. Since the end-product will contain traces of residual solvent, the solvent should be non-toxic and not interfere with the bioassay.[96]

Various other factors, such as the extraction period and temperature, could influence the products of extraction.[97, 98] This was observed by Dent and colleagues in their study to examine the effects of extraction parameters, such as solvent polarity,

temperature and extraction time on sage polyphenols.[98] The mass fractions of sage TPP compounds (rosmarinic acid and luteolin-3-glucuronide), as well as other sage polyphenols, varied considerably as a function of the type of solvent (ethanol, acetone or water), solvent composition (water/organic solvent) and extraction temperature, while extraction time had a significant influence on the mass fraction of luteolin-3-glucuronide.[98]

2.1.3. Phytochemical screening of plant extracts

Plant extracts are made up of a cocktail of different phytochemical groups such as alkaloids, anthraquinones, essential oils flavonoids, glycosides, phenols, saponins, steroids tannins, and terpenes, with each linked to certain pharmacological activities.[99] Total alkaloids extracted from *Rubus alceifolius* were reported to inhibit angiogenesis in a chick embryo chorioallantoic membrane assay, and inhibited proliferation of human umbilical vein endothelial cells.[100] A secondary alkaloid, 4-carboethoxy-6-hydroxy-2-quinolone, from *Oryza sativa* showed significant antioxidant activity against the DPPH radical.[101] Furthermore, the inhibitory effect of *Stylissa massa* against biofilm formation in Gram-negative bacteria has been attributed to dibromoisophakellin and dibromophakellin, which are bromopyrole alkaloids.[102]

Phenolic compounds, one of the largest groups of secondary metabolites from plants, also exhibit a series of biological properties that promote the health needs of man, including wound management. These include flavonoids such as catechin, kaempferol, luteolin, and quercetin; phenolic acids: caffeic acid, coumaric acid, gallic acid, syringic acid, and vanilic acid; tannins like tannic acid; and stilbenes such as resveratrol (Figure 13). These secondary metabolites are known to have diverse medicinal benefits, including antimicrobial, antioxidant, and anti-inflammatory properties, which could be useful for the promotion of wound healing.[103] As a result of the numerous medicinal properties of phytochemical components, various scientific methods, such as chromatography and electrophoresis are employed in the determination of the specific compounds or chemical groups responsible for the observed effects of medicinal plants.[99, 104]

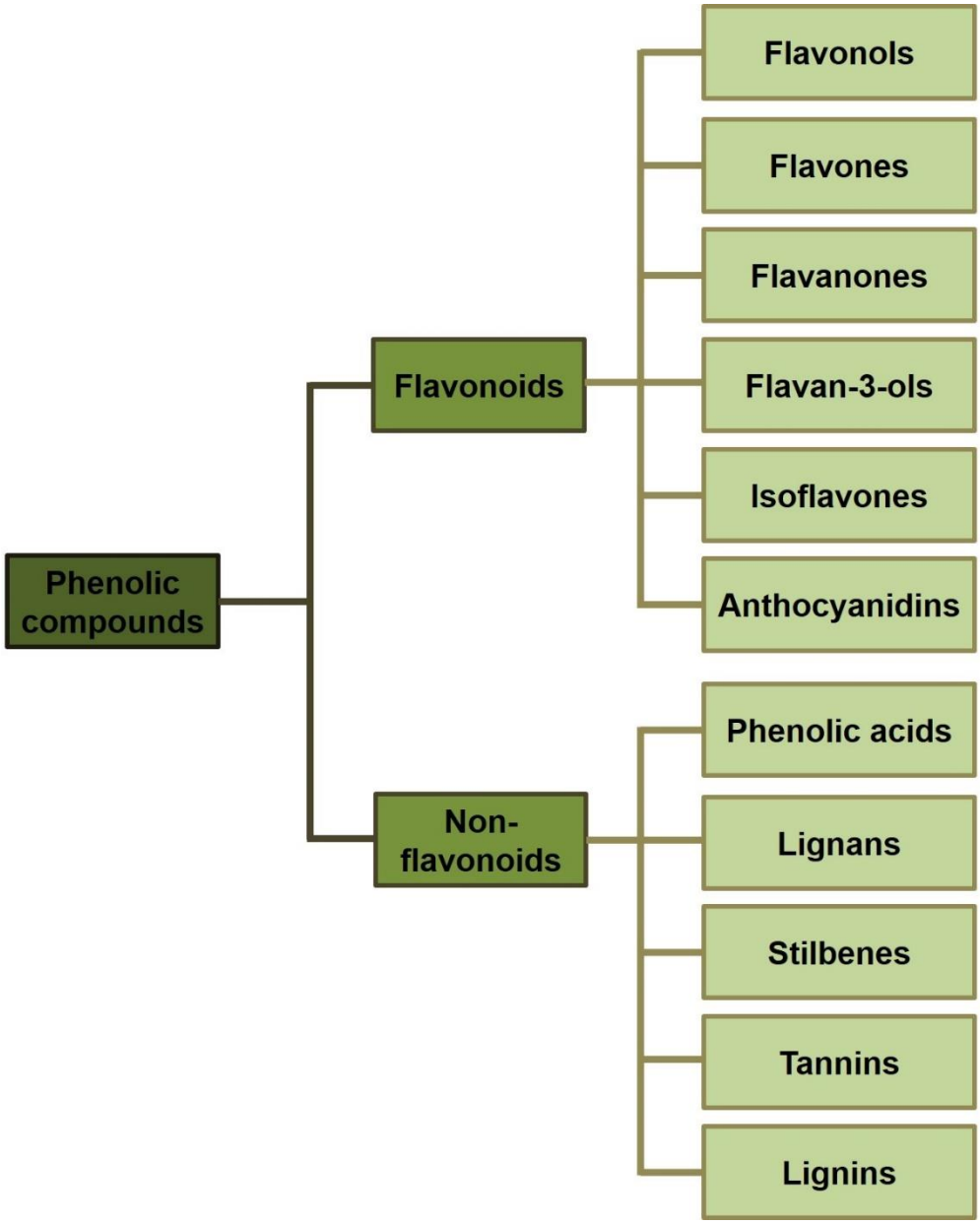

Figure 13: Classification of phenolic compounds (adapted from Dzialo *et al*.).[103]

2.1.4. Separation methods

Plant extracts are made up of a complex mixture of phytochemical compounds. The identity of the specific compounds responsible for biological activities involves the separation of the constituents. For this purpose, various chromatographic techniques are employed. These include paper chromatography (PC), TLC, open-column chromatography (CC), vacuum-liquid chromatography, high performance liquid chromatography (HPLC), and liquid-liquid chromatography. All of them have their own

advantages and limitations, and therefore best results are obtained by combination of two or more different methods.

All chromatographic techniques are based on the same principle of separating two or more compounds from a mixture by distributing between the mobile and stationery phases.[104] Paper chromatography is particularly suitable for separating water-soluble compounds, including amino acids, carbohydrates, organic acids, and phenolic compounds. It is a simple and convenient method which separates mixtures by use of paper (stationery phase), and eluting with a suitable solvent (mobile phase). This method is relatively cheap, works well with crude extracts, and results in separations with considerably reproducible retention factors (Rf), a valuable parameter for use in describing new compounds.[105] Its major disadvantage is the relatively limited resolving power. Some mixtures of related compounds are difficult to separate by this method, if not impossible.[105]

Thin layer chromatography, on the other hand is the method of choice for separation of lipid-soluble components such as carotenoids, isoflavonoids lipids, quinones, and steroids. The separation of hydrophilic components by TLC is achieved by use of polyamide or microcrystalline cellulose plates.[104, 105] This method provides greater flexibility in choice of solvent, has improved separation speed, and better retention power, compared to PC. Detection of compounds on TLC is achieved by spraying with specific spray reagents and processed under relevant conditions for compound-specific colour development. Column chromatography is very similar to TLC, the difference being the size of stationery phase. In TLC, the stationery phase is a thin layer of silica gel or alumina on a glass, metal or plastic plate.[104] For CC, the same stationery phase is packed into a vertical glass column, and thereby enabling the separation of larger amounts of mixtures.

High performance liquid chromatography enables qualitative and accurate quantitative analysis of samples. Unlike the other chromatographic methods, HPLC allows for analysis of a wide range of substances, including carbohydrates, flavonoids, lipids, and other phenolic compounds, as well as amino acids. It differs from CC with the presence of a pump which drives the mobile phase through the system under pressure; an injector, for injecting samples; and its direct linkage to a detecting device.

Depending on the nature of the stationary phase, different separation categories are available; adsorption chromatography, partition chromatography, ion-exchange chromatography, and size exclusion chromatography.[105, 106] The choice of the mobile phase depends on the nature of the operation mode, that is, isocratic or gradient elution. The polarity for such an elution medium can, therefore, vary from buffered aqueous solutions to hydrocarbons.[106] Ultra performance liquid chromatography is a relatively new technique which thrives on the principles of HPLC. However, this has superior speed, resolution, and sensitivity, compared to HPLC.[107]

Another commonly used technique for separation of compounds is electrophoresis. This is, however, only useful for separating amino acids, organic acids, alkaloids, and compounds that can be made to move in an electric field by virtue of their charges.[104] In this study, a combination of TLC and UPLC was used to evaluate the identity of phytochemicals present.

2.1.5. Methods of identification

Separated components of extracts can be identified using colour tests, chromatographic procedures, and spectral measurements.[105] Although each identification method is unique, a combination of one or more techniques is often required for accurate identification of compounds. Whilst the class of compounds can readily be identified from biochemical and colour tests with chromogenic reagents, additional tests are required for the identification of specific compounds.[104] For instance, reducing sugars produce a colour reaction with Fehling's reagent, whilst alkaloids form a reddish-brown or orange precipitate with Dragendorff's reagent. Other colour reagents and the specific groups of compounds they detect are listed in Section 2.6.

Much can be learnt from the chromatographic data of a compound. Examination of a developed chromatogram from PC or TLC under ultra violet (UV) light reveals identity of some groups of compounds, as not all compound spots can be detected using the visible spectrum of light.[104] Coumarins for example, produce a characteristic blue fluorescence at 360 nm. Furthermore, spraying the developed chromatogram with spray reagents produces coloured spots which reveal the identity of phytochemical groups. Spraying a developed chromatogram with Dragendorff's reagent, for example,

reveals the presence of alkaloids as orange spots. Moreover, measurement of the Rf value of the spots on a chromatogram could reveal the identity of the compound.[108] This is achieved by comparing the measured Rf with published values of known compounds.

Data from advanced techniques such as HPLC/UPLC can more reliably reveal the identity of compounds. This produces peaks, with characteristics such as retention times, wave lengths, and peak heights, which are unique for each compound.[107] Compounds are identified by comparing the peak characteristics with that of an already known one such as phytochemical standards. This can be achieved by injecting standard solutions under identical analytical conditions. Comparing the retention time and response of the peak in the chromatogram of the standard solution with the sample chromatogram may reveal the identity of compounds responsible for the unknown peaks. Interestingly, the peak area or size can be used to quantitatively determine the concentration of sample present.

To greatly increase confidence in peak assignment, detector systems such as diode array UV spectrometers or mass spectrometers (MS) are used in tandem with HPLC/UPLC to record unique spectra for each peak within the sample chromatogram.[104, 105] The spectra obtained from the test sample peak are compared with a spectrum from either a standard solution of pure compound run alongside the sample, or with reference spectra. Other spectral measurements such as infrared and nuclear magnetic resonance (NMR) are equally useful for compound identification.

In this study, chemical groups were identified by spraying developed TLC plates with various chromogenic spray reagents, and visualizing under visible and UV light. Chromatographic fingerprints were also obtained for each plant extract using UPLC and MS, and peaks compared with that of standard compounds.

2.2. Aim and objectives of chapter

This chapter was aimed at preparing the extracts and assessing their phytochemical composition.

The objectives were to:

- prepare sequential and ethnomedicinal extracts from *A. africana, B. diffusa* and *E. senegalensis* using hexane, ethyl acetate, methanol and water as solvents
- determine the phytochemical composition of the extracts using TLC
- obtain chromatographic fingerprints from all extracts using UPLC-qTOF-MS.

2.3. Materials and Methods

Ethical approval for the conduct of the research was obtained from the Research Ethics Committee of the University of Pretoria Faculty of Health Sciences, with reference number 194/2017 (Appendix II). A list of the reagents and solutions used is provided in Appendix III.

2.3.1. Plant material

2.3.1.1. Plant collection

Whole plants of *A. africana* and *B. diffusa*, and leaves of *E. senegalensis,* were collected from the botanical garden of the University of Cape Coast, Ghana (GPS coordinates; 5º7'4.251"N 1º17'36.347"W). The identity of the specimens was authenticated at the University's School of Biological Sciences herbarium, where voucher specimens are deposited for *A. africana* (UCCH 0211215), *B. diffusa* (UCCH 0041215), and *E. senegalensis* (UCCH 0091215). The samples were washed thoroughly, air-dried at room temperature, finely powdered with a grinder (Glen Creston, UK) and stored in sterile airtight containers.

2.3.1.2. Preparation of extracts

Extraction was performed sequentially with four different solvents in increasing polarity as described by Arokiyaraj *et al*.[109] 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. Samples were filtered through a 0.22-µm Millipore filter paper under vacuum pressure, and filtrates (except water extract) dried *in vacuo* with a rotary evaporator (Buchi Rotavapor R-200) at 40ºC. Filtrates obtained from water extraction were freeze-dried (Freezone 6 Freeze Dry System, Labconco 31). Gravimetric yield for each extract was determined as a percentage of the dry weight of extracts and the plant material extracted. Organic extracts (50 µL) were evaporated on pre-weighed petri dishes, and their dry weight determined. The organic extracts were reconstituted in dimethyl sulfoxide (DMSO) and 10 mg/mL aliquotes stored at -20ºC until needed. Lyophilized water extracts were stored in air-tight containers at -20ºC.

Although most traditional healers employ heat in extraction, hot water extracts were not prepared in this study because of the potential effect of high temperatures on compounds present.[97] Furthermore, these medicinal plants are often used topically for the management of wounds, a practice which does not involve boiling. Therefore, ethnomedicinal extracts were prepared from each plant using distilled water. Powdered plant material (10 g) was macerated in 100 mL distilled water, shaken for 30 min on an electronic shaker, and incubated at room temperature for 24 h. The solvent was decanted, filtered, freeze-dried, and stored in air-tight containers at -20ºC as described above for the water extracts.

2.3.2. Phytochemical analysis

Thin-layer chromatography was used to screen extracts for the presence of alkaloids, coumarins, flavonoids, glycosides, phenols, tannins, terpenes and steroids with the aid of various mobile phases and detection reagents (Table 3). The extracts were dissolved in methanol to a concentration of 10 mg/mL, and the various chemical constituents separated from 10 µL aliquots using aluminium-backed silica gel 60 F_{254} TLC plates (Merck, SA). Plates were prepared in triplicate, and developed using various eluent systems as described previously.[110] Separated components were visualized under ultraviolet light (254, 360 nm) using a Camag Universal UV lamp TL-600. Plates were sprayed with selective visualisation reagents (Table 3), and heated to allow for development of colour changes.[111]

Table 3: Thin-layer chromatography mobile phases and spray reagents.[108]

Class	Mobile phase	Spray reagent	Colour indicator
Alkaloids	Benzene:chloroform:methanol (7:2:1)	Dragendorff's reagent	Orange coloured spots
Saponins	Benzene:methanol (9:1)	Anisaldehyde-sulphuric acid reagent	Blue/green fluorescence at 360 nm
Coumarins	Benzene:methanol (9:1)	Potassium hydroxide reagent	Blue fluorescence at 360 nm
Flavonoids	Chloroform:methanol:butanone (9:4:2)	1% ethanolic solution of aluminium chloride	Yellow fluorescence at 360 nm
Phenols	Chloroform:methanol:butanone (9:4:2)	Folin-Ciocalteu reagent	Blue coloured spots
Glycosides	Benzene:chloroform:methanol (7:2:1)	Anisaldehyde-sulphuric acid	Violet spots at 110ºC
Tannins	Benzene:methanol (9:1)	Iron-III-chloride reagent	Blue-black spots
Terpenes	Benzene:methanol (9:1)	Anisaldehyde-sulphuric acid	Blue spots at 105ºC
Steroids	Benzene:methanol:acetic acid (79:14:7)	Anisaldehyde-sulphuric acid	Green spots at 105ºC

2.3.3. High performance liquid chromatography fingerprinting

A Waters UPLC coupled in tandem to a Waters photodiode array (PDA) detector and a SYNAPT G1 High Definition Mass Spectrometer (HDMS) was used to generate accurate mass data. Optimization of the chromatographic separation was done using a Waters HSS T3 C18 column (150 mm × 2.1 mm, 1.7 µm) that was maintained at 60ºC. All solvents used for the UPLC-MS experiments were of ultrapure LC-MS grade. A binary solvent mixture consisting of water (eluent A) containing 10 mM formic acid (natural pH of 2.3) and acetonitrile (eluent B) containing 10 mM formic acid was used. The initial conditions were 100% eluent A at a flow rate of 0.4 mL/min, which was maintained for 1 min, followed by a linear gradient to 5% eluent A at 16 min. The conditions were kept constant for 1 min and then changed back to the initial conditions. The run time was 20 min and the injection volume set at 3 µL, with triplicate injections acquired per sample. The PDA detector was scanned between 200 and 500 nm (1.2 nm resolution) while collecting 20 spectra per second.

The SYNAPT G1 HDMS was used in V-optics and operated in electrospray mode to enable detection of phenolic and other electro spray ionization (ESI)-compatible compounds. Leucine enkephalin (50 pg/mL) was used as reference calibrant to obtain typical mass accuracies between 1 and 5 mDa. Although positive ionization is

commonly used and more compounds are ionized in this state,[112] the mass spectrometer was operated in both negative and positive modes of electrospray ionization to enable greater coverage of the metabolome for untargeted analysis. The capillary voltage was set at 2.5 kV, the sampling cone at 30 V, and the extraction cone at 4.5 V. The scan time was 0.1 s covering the 100 to 1400 Da mass range. The source temperature was 120ºC and the desolvation temperature was set at 450ºC. Nitrogen gas was used as the nebulization gas at a flow rate of 550 L/h, and cone gas was added at 50 L/h. MassLynx 4.1 (SCN 872) software was used to control the hyphenated system as well as for data manipulation.

Experimental samples, made up of the plant extracts and previously identified compounds in the plants (standards), were re-dissolved in methanol to a concentration of 1 mg/mL (standards) and 10 mg/mL (extracts), and filtered through a 0.2 µm pore size filter paper. The standard compounds (Table 4) were first run through the UPLC-qTOF-MS system to obtain reference fingerprints for the respective extracts.

Table 4: Phytochemical standards used for UPLC-qTOF-MS analysis of extracts

Sample	Compound/s	Reference
Aspilia africana	Limonene Ascorbic acid	Kuiate *et al*.,[113] Okwu and Josiah[76]
Boerhavia diffusa	Quercetin Kaempferol Rutin	Ferreres *et al*.,[86] Patil and Bhalsing[78]
Erythrina senegalensis	Neobavaisoflavone	Kuete *et al*.[114]

2.4. Statistical analysis

Experiments were carried out using a minimum of three technical and biological repeats. The data for yield determination was presented as mean ± standard error of mean (SEM), and analysed using the Graphpad software (Graphpad Prism 7).

2.5. Results and discussion

2.5.1. Yield of plant extracts

Plant samples were extracted sequentially using four different solvents, as well as ethnomedicinally using water. Although most traditional healers use water to prepare

their decoctions because it is readily available, a major disadvantage of using water for extractions is its limited ability to extract non-polar compounds. The type of solvent used determines the products of extraction.[115] Different solvents with varying polarity were used in this study in order to extract a broad range of compounds from the plant samples. Generally, the extraction yield in the polar solvents was much higher, compared to the less polar solvents (Table 5). The yield ranged from 1.40% in the hexane fraction of *A. africana* to 22.60% in the methanol fractions of *E. senegalensis*. Comparatively, though the yield of the three medicinal plants was higher with methanol, the ethnomedicinal extract yields were relatively high also, with a range between 6.90% and 13.92% for *E. senegalensis* and *B. diffusa*, respectively. These were much higher than the yield obtained for their corresponding water fraction from the sequential extraction. This may be due to sequential removal of phytochemicals during the hexane, ethyl acetate and methanol extraction steps.

Table 5: Extraction yields of the sequential and ethnomedicinal prepared extracts.

Plant sample	Part extracted	Extract	Yield (% w/w)
Aspilia africana	Whole plant	H	1.40
		E	2.60
		M	14.60
		W	5.80
		ET	11.10
Boerhavia diffusa	Whole plant	H	2.60
		E	4.00
		M	21.40
		W	11.52
		ET	13.92
Erythrina senegalensis	Leaves	H	2.60
		E	6.60
		M	22.60
		W	5.60
		ET	6.90

H, hexane extract; E, ethyl acetate extract; M, methanol extract; W, water extract; ET, ethnomedicinal extract

2.5.2. Phytochemical analysis

Extracts prepared from natural products such as plants possess a complex and variable mixture of chemicals known as phytochemicals. Phytochemical entities such as the major plant secondary metabolites alkaloids, phenols and terpenes have diverse pharmacological properties, both therapeutic and adverse. For instance,

alkaloids are known for their antioxidant and antimicrobial potential,[102] and the anti-inflammatory and wound healing activities of phenolic compounds have also been reported.[43] Qualitative analysis of extracts for the presence of these major phytochemical entities was done with the aid of TLC using standard spray reagents and visualized under UV and visible lights.

2.5.2.1. *Aspilia africana*

Alkaloids were present in all the extracts of *A. africana*, except the water extract (Table 6). Each extract possessed at least three of the phytochemicals tested, with the methanol extract testing positive for all the chemical classes. Steroids were only detected in the methanol extract, whereas the ethyl acetate and methanol extracts tested positive for tannins. In general, the phytochemical groups of *A. africana* were predominantly extracted into the more polar solvents. The findings are largely in agreement with that of previous studies conducted on the plant. Whilst Okoli and colleagues tested for presence of the phytochemicals in the methanol extract of the leaves, a more recent study did so using aqueous extracts from the flowers.[74, 116] Though the latter study detected steroids in the aqueous extract, the former and current studies only detected steroids in the methanol extract. This could be due to the difference in plant parts used, suggesting a varying phytochemical composition in different parts of the plant.

Table 6: Phytochemical classes detected in extracts of *A. africana* by TLC

Phytochemical	H	E	M	W	ET
Alkaloids	+	+	+	-	+
Flavonoids	+	-	+	+	+
Glycosides	-	+	+	-	+
Phenols	+	-	+	+	+
Saponin	-	-	+	-	-
Steroid	-	-	+	-	-
Tannins	-	+	+	-	-
Terpenes	-	-	+	+	+

H, hexane extract; E, ethyl acetate extract; M, methanol extract; W, water extract; ET: ethnomedicinal extract; (+) constituent present; (-) constituent absent.

2.5.2.2. *Boerhavia diffusa*

All extracts of *B. diffusa* were found to contain alkaloids, flavonoids, and phenols (Table 7). The ethyl acetate extract had all phytochemical groups present, except for glycosides, steroids, and terpenes, whereas the only groups present in the water extract were alkaloids, flavonoids and phenols. Interestingly, none of the *B. diffusa* extracts tested positive for steroids, and tannins. This is contrary to published reports by Apu *et al.*, where the presence of tannins in the ethyl acetate and methanol crude extracts of the aerial parts of the plant has been described.[117] The current and previous study are however in agreement with regards to the presence of the other constituents. Differences in findings could be due to the different extraction procedures used, as well as collection site. Whilst samples in the present study were extracted sequentially using different solvents, that of Apu *et al*. involved the use of crude preparations.[117] Furthermore, their samples were collected from Bangladesh, whereas plant samples for the present study were collected from Ghana. According to earlier accounts, same plant species collected from different sites could have variations in chemical composition.[86]

Table 7: Phytochemical classes detected in extracts of *B. diffusa* by TLC.

Phytochemical	H	E	M	W	ET
Alkaloids	+	+	+	+	+
Flavonoids	+	+	+	+	+
Glycosides	-	-	+	-	+
Phenols	+	+	+	+	+
Saponin	-	+	+	-	-
Steroid	-	-	-	-	-
Tannins	-	-	-	-	-
Terpenes	-	+	+	-	-

H, hexane extract; E, ethyl acetate extract; M, methanol extract; W, water extract; ET: ethnomedicinal extract; (+) constituent present; (-) constituent absent.

2.5.2.3. *Erythrina senegalensis*

The *E. senegalensis* extracts were found to contain the highest number of phytochemical classes (Table 8). Each phytochemical class tested for was detected in at least one of the extracts. All five extracts assessed possessed phenolic compounds, whilst saponins and steroids were only detected in the methanol extract. Alkaloids, flavonoids, and phenols were found in at least four of the extracts tested.

The polar extracts of *E. senegalensis* also tested positive for more phytochemical components than the least polar ones. Similar findings were obtained by Temitope and colleagues.[118] In their account, steroids were present in the ethyl acetate crude extracts of the leaves collected from Nigeria.[118] In the present study however, steroids were only detected in the methanol sequential extract. This could be due to differences in extraction procedure used, as well as the nature of steroids present.[86]

Table 8: Phytochemical classes detected in extracts of *E. senegalensis* by TLC.

Phytochemical	H	E	M	W	ET
Alkaloids	-	+	+	+	+
Flavonoids	+	+	+	-	+
Glycosides	-	+	+	-	+
Phenols	+	+	+	+	+
Saponin	-	-	+	-	-
Steroid	-	-	+	-	-
Tannins	+	+	-	-	-
Terpenes	-	-	+	+	+

H, hexane extract; E, ethyl acetate extract; M, methanol extract; W, water extract; ET: ethnomedicinal extract; (+) constituent present; (-) constituent absent.

2.5.3. UPLC-MS fingerprinting

To confirm the identity of the plants, fingerprints were obtained for each extract using UPLC-qTOF-MS. The presence of certain secondary metabolites that have previously been reported as being present in the plants (marker compounds) were also determined using the same technique. Marker compounds included ascorbic acid, kaempferol, quercetin, neobavaisoflavone, rotenone, rutin, and limonene (Figure 14; mass chromatograms of these standard compounds are provided in Appendix IV).

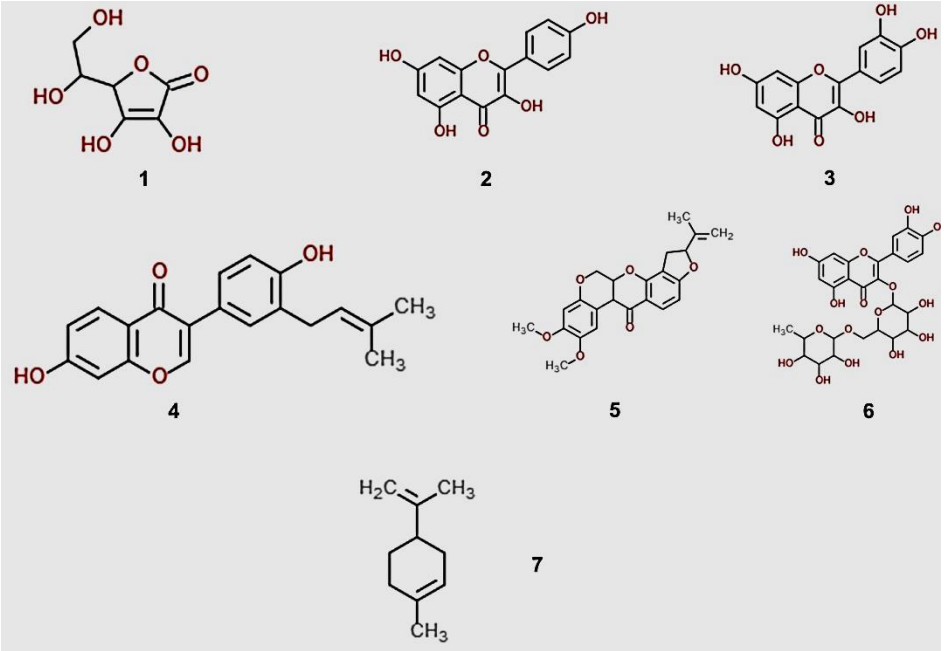

Figure 14: Phytochemical standards used for fingerprinting of extracts. Ascorbic acid, **1**; kaempferol, **2**; quercetin, **3**; neobavaisoflavone, **4**; rotenone, **5**; rutin, **6**; limonene, **7**

2.5.3.1. *Aspilia africana*

A. africana possessed diverse peaks, as shown by UPLC-qTOF-MS (Figure 15), indicating the presence of several compounds. Many prominent peaks were observed, such as ascorbic acid (m/z = 175.023), which was present in the hexane, water and ethnomedicinal extract. Also present in the ethnomedicinal extract was a peak with a retention time of 5.71 min and m/z ratio of 305.108 which corresponds to quercetin or one of its derivatives. None of the marker compounds tested was detected in the ethyl acetate and methanol extracts.

Quercetin and its derivatives such as quercetin-3-O-α-L-rhamnosyl(1-6)-β-galactopyranoside and quercetin-3,7- di-O-glucoside are phenolic compounds known as flavonol glycosides.[78] These were detected in the ethnomedicinal extract of *A. africana*, an extract which tested positive for flavonoids and phenolic compounds using TLC (Table 6). Although the hexane, methanol and water extracts of the plant also tested positive for these phytochemical classes, quercetin was not detected. This would suggest the presence of other flavonoids and phenolic compounds.

To the best of the authors knowledge, this is the first study evaluating the chemical composition of *A. africana* using UPLC-MS. All other assessments either involved biochemical evaluations[76] or GC-MS.[113] Ascorbic acid,[76] germacrene-D, limonene, and α-pinene,[113] have been reported to be present in the leaves of *A. africana*. Limonene could not be detected in any of the extracts, possibly due to differences in sampling location, extraction techniques or analytical methods.

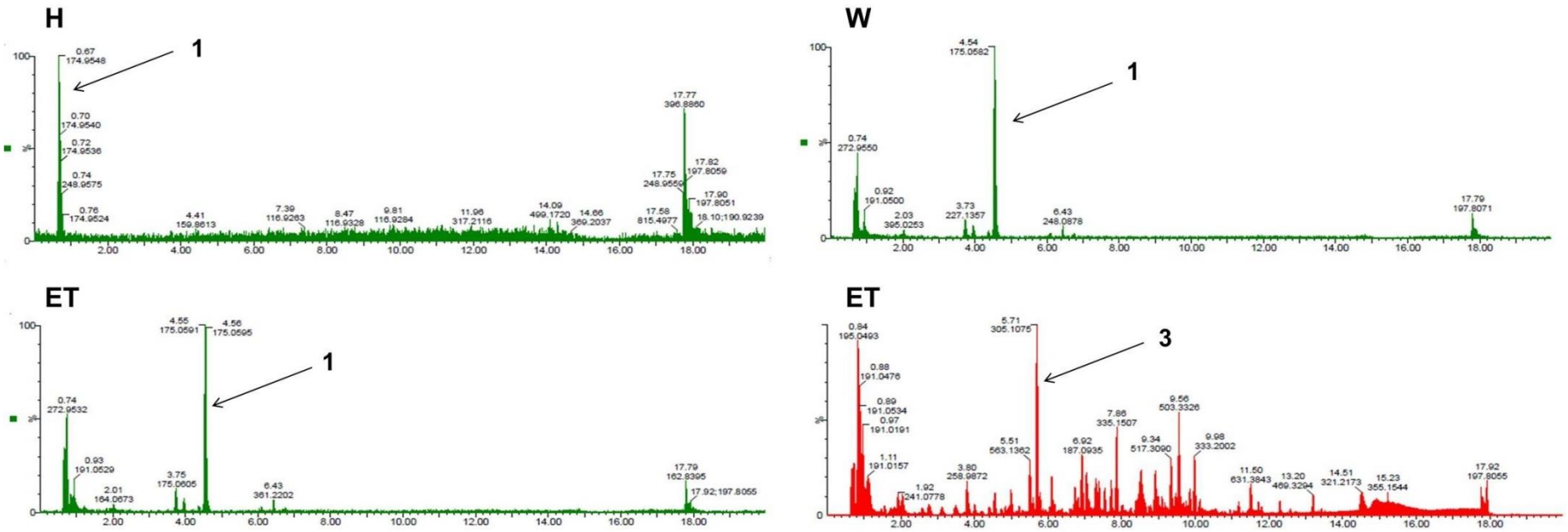

Figure 15: Mass chromatograms of *A. africana* hexane (H), water (W), and ethnomedicinal (ET) extracts. All data are presented in ESI-negative mode. Ascorbic acid, **1**; quercetin**, 3**

2.5.3.2. *Boerhavia diffusa*

The UPLC-MS analysis of *B. diffusa* extracts revealed the presence of various chemical entities (Figures 16 and 17). Kaempferol (m/z = 285.042), or its derivatives, was detected in all extracts, except the water extract. Also, quercetin (m/z = 301.032), or its derivatives, was detected in all but the ethyl acetate extract. On the other hand, the methanol and ethyl acetate extracts tested positive for rutin (m/z = 609.145; Figure 16). None of the other marker compounds tested were detected in the extracts. All extracts of the plant tested positive for flavonoids and phenols, the compound classes detected during TLC analysis (Table 7). However, none of the standard compounds were detected in the water extract. This suggests the presence of other phenolic compounds and flavonoids.

Limonene, kaempferol, quercetin, and rotenone, have all previously been reported to be present in extracts of *B. diffusa*.[78, 86] Though rutin has not previously been detected in this plant, its presence in another species of the same genus (*B. erecta*) is well known.[78] The detection of rutin in extracts used in this study may partly be ascribed to the superior sensitivity of UPLC compared to HPLC and other chromatographic techniques used in the previous studies. Also, since neither of the previous studies was conducted using the extraction technique employed in the present study or samples collected from Ghana, these could contribute to the difference in findings. This study has therefore shown that in addition to the other detected compounds, part of the therapeutic potential of extracts from *B. diffusa* could be a result of the presence of rutin.

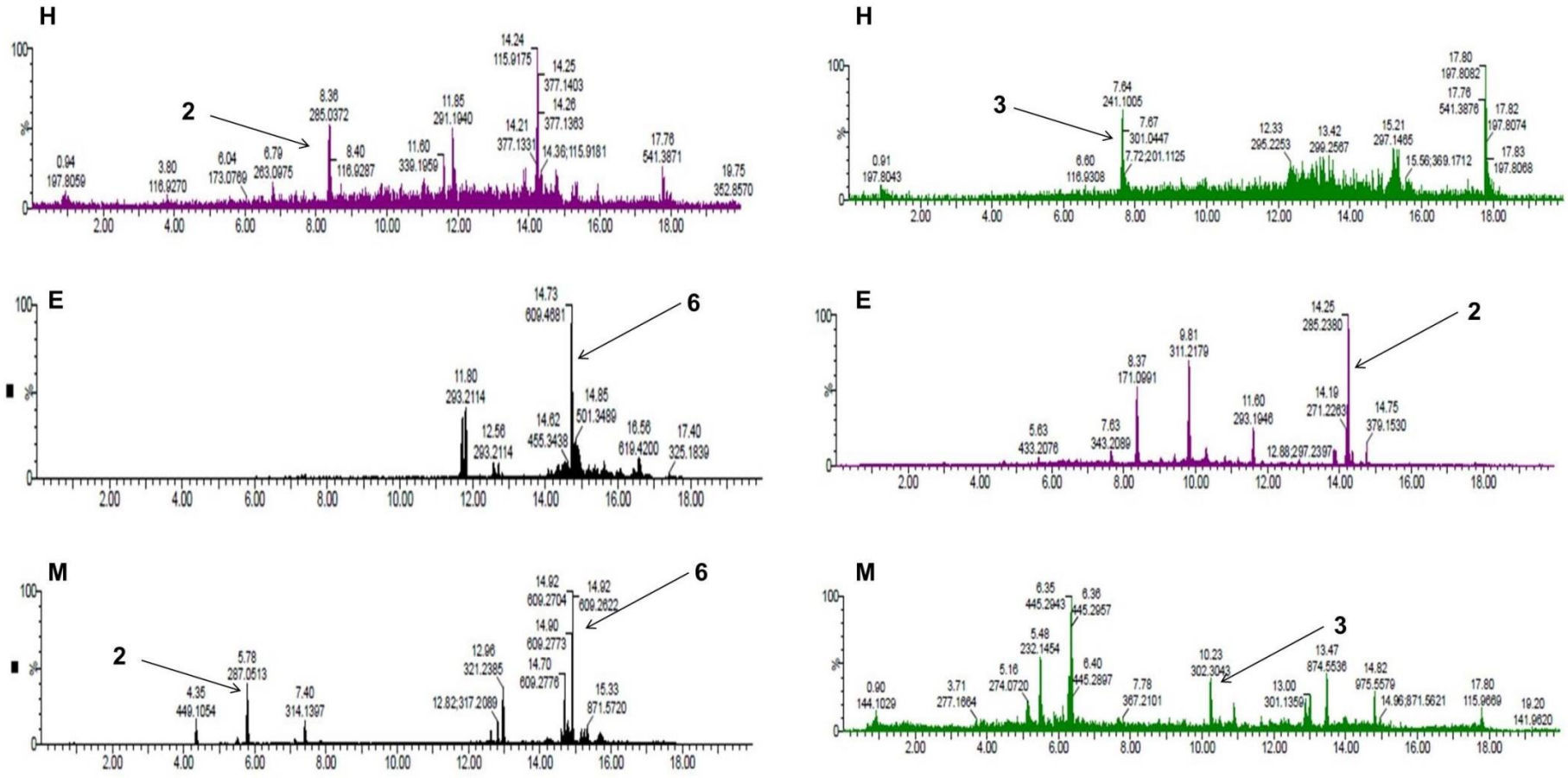

Figure 16: Mass chromatograms of *B. diffusa* hexane (H), ethyl acetate (E), and methanol (M) extracts. All data are presented in ESI-negative mode. Kaempferol, **2**; quercetin, **3**; rutin, **6**

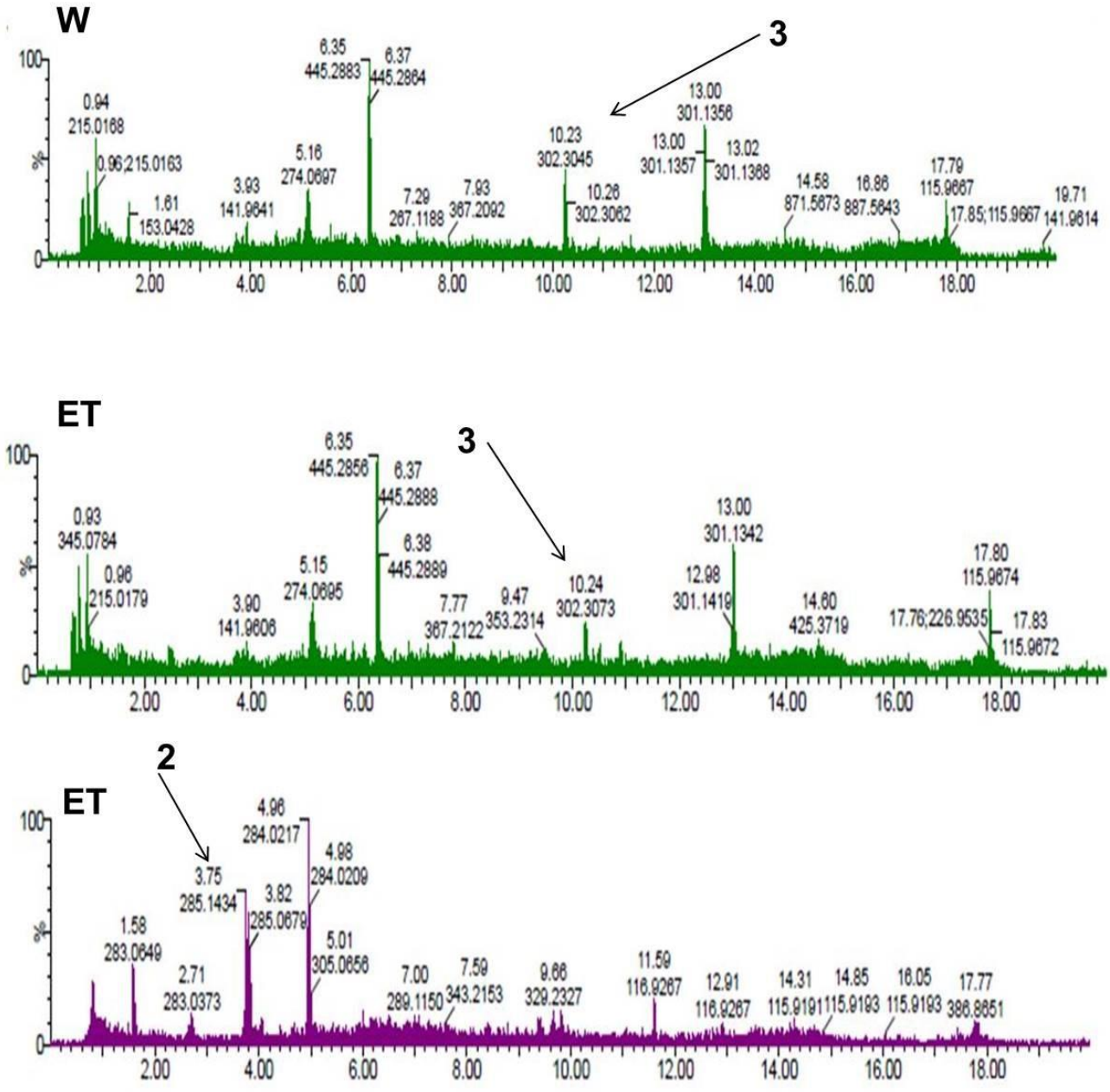

Figure 17: Mass chromatograms of *B. diffusa* water (W) and ethnomedicinal (ET) extracts. All data are presented in ESI-positive mode. Kaempferol, **2**; quercetin, **3**

2.5.3.3. *Erythrina senegalensis*

E. senegalensis was the most complex of all the three samples studied as depicted by the numerous compounds detected upon UPLC-MS analysis (Figures 18 and 19). Four of the marker compounds tested, kaempferol, neobavaisoflavone, rotenone, and rutin, were detected in extracts prepared from the plant. Neobavaisoflavone (m/z = 321.112) was detected in the hexane, ethyl acetate, and methanol extracts, whereas kaempferol (m/z = 285.042) and rotenone (m/z = 393.132) were only detected in the methanol extract. Also, rutin (m/z = 609.145) was detected in the ethyl acetate and methanol extracts. None of the compounds mentioned were detected in the water extract of the plant. However, traces of neobavaisoflavone were detected in the ethnomedicinal extract (not indicated on chromatogram due to low levels).

All the marker compounds of this plant species detected were isoflavonoids, which are all phenolic compounds. Furthermore, these phytochemical groups were shown to be present in the extracts by TLC (Table 8). Of the four compounds detected, only neobavaisoflavone has previously been reported.[114] Many studies have been conducted on the plant with the aim of determining its phytochemical constituents, however, none employed UPLC-MS. The latter, together with the differences in extraction techniques and sampling sites could account for the difference in findings. Also, since there is no mention of absence of the marker compounds tested in previous publications, it could also be that previous research was not focused on these compounds. It therefore would appear as if this is the first study to indicate the presence of kaempferol, rotenone, and rutin, in the leaves of *E. senegalensis*.

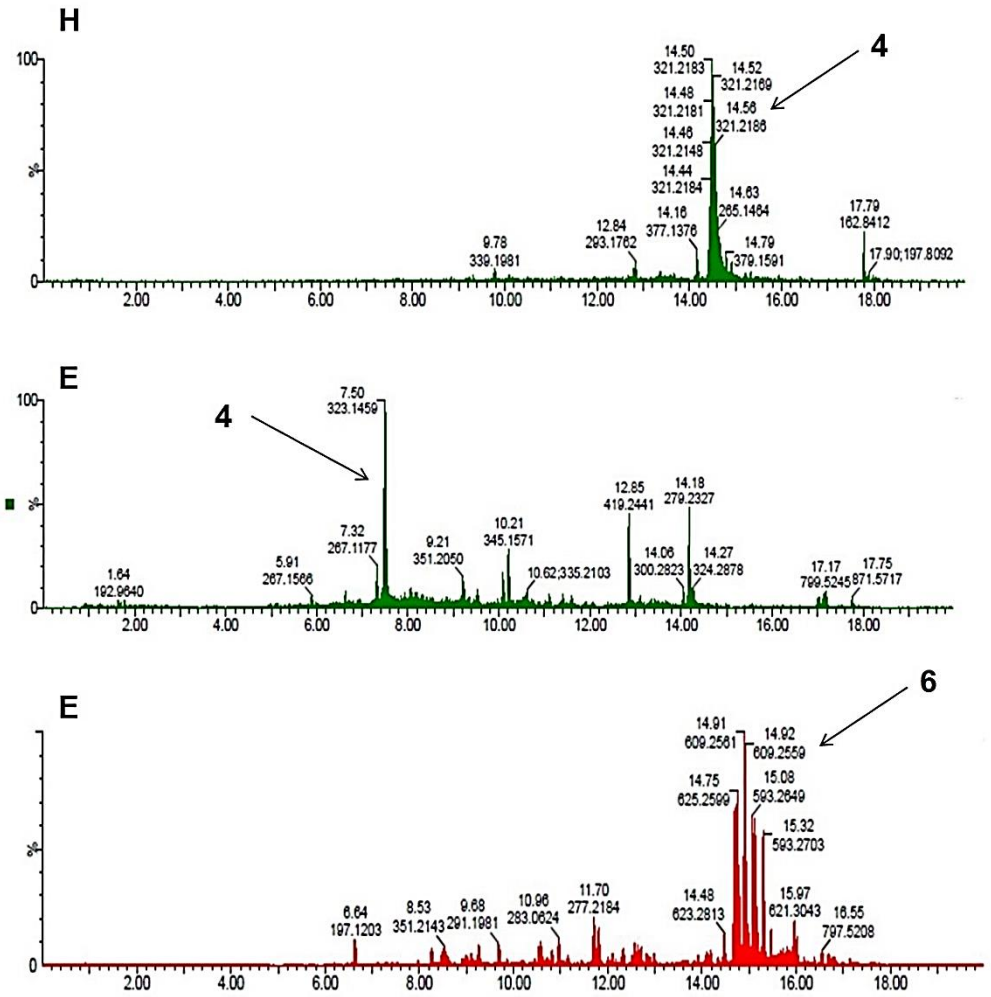

Figure 18: Mass chromatograms of *E. senegalensis* hexane (H) and ethyl acetate (E) extracts. Data for the hexane extract are presented in ESI-negative mode, whilst the ethyl acetate extract was run in ESI positive mode. Neobavaisoflavone, **4**; rutin, **6**

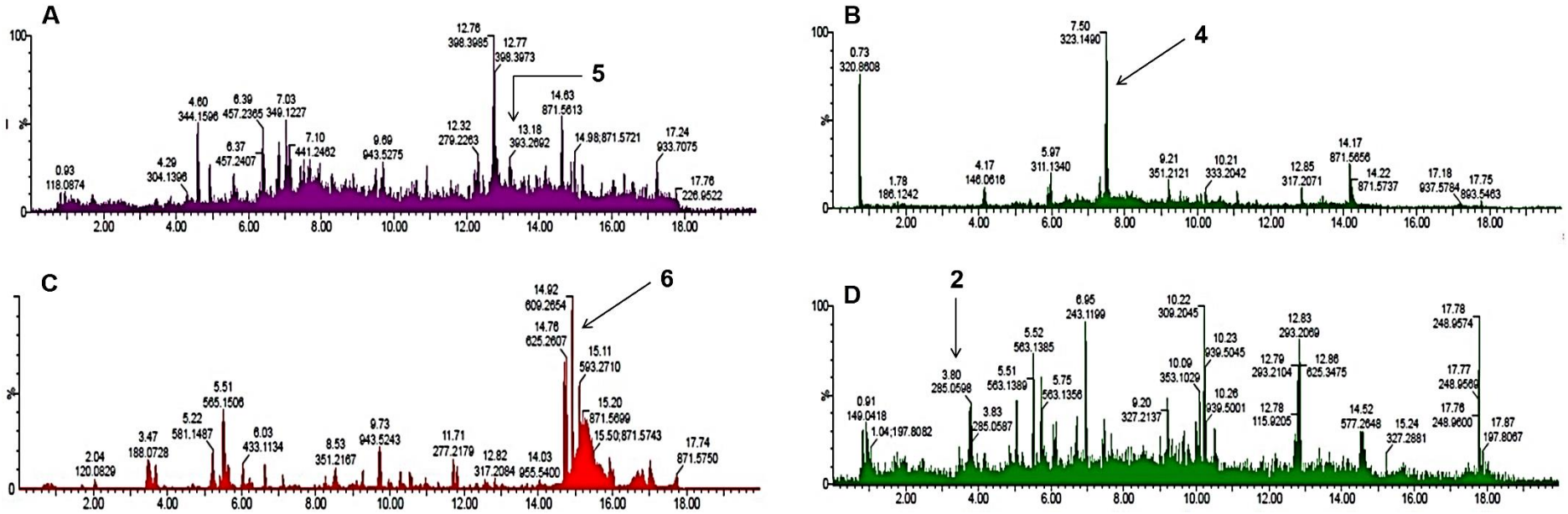

Figure 19: Mass chromatograms of *E. senegalensis* methanol extract. A-C are in ESI-positive mode, D is in ESI-negative mode. Kaempferol, **2**; neobavaisoflavone, 4; rotenone, **5**; rutin, **6**

2.6. Conclusion

The plants were successfully extracted using the sequential extraction technique and an ethnomedicinal method which mimicked the traditional way of extract preparation. Analysis of extracts for major phytochemical groups using TLC showed that each solvent extract possessed at least one of the major phytochemical groups, suggesting extraction of multiple phytochemical entities. Several phytochemicals were identified for the first time in the plants using UPLC-qTOF-MS. These include quercetin in the ethnomedicinal extract of *A. africana*, and rutin in the ethyl acetate and methanol extracts of *B. diffusa*. Additionally, kaempferol, rotenone, and rutin were detected for the first time in various extracts of *E. senegalensis*. Whilst this could be due to the superior analytical technique employed in this study compared to previous studies on the three plants, the sequential extraction approach used could also contribute to increasing the detectability of the compounds.

Chapter 3

Cytotoxicity evaluation

3.1. Introduction

3.1.1. Relevance of cytotoxicity evaluations

Determination of cytotoxicity of chemical substances, including herbal extracts, is an important step in pre-empting potential toxic manifestations. It also enhances our understanding of the hazardous effects of chemicals and predicting the same in humans. In the words of Paracelsus, “all things are poison and nothing is without poison, only the dose permits something not to be poisonous”.[119] This statement received world-wide attention in the early 1960s from what is referred to as the thalidomide disaster, where thousands of babies were born with debilitating birth defects as a result of drug treatment in pregnant patients.[120] Disasters such as this could be detected and avoided through toxicity evaluations in the early stage of the drug development process.

Currently, several people world-wide use herb-based medications for maintenance of healthcare.[121] These products are often perceived as safe because of their natural origin. However, numerous cases of herb-induced toxicity have been reported. Various plant species, including *Glycyrrhyza glabra*, and *Lysimachia paridiformis*, are known for their cytotoxic potential because of the presence of secondary metabolites such as saponins.[122] This metabolite has also been linked to toxicity induced by species from numerous other plant families such as Agavaceae, Dioscoreaceae, and Liliaceae.[122] *Aloe sp.*, commonly known for its positive effects on wound healing, and other dermatological conditions, has been linked to the induction of a toxic hepatitis in patients with known use of oral preparations from the plant.[123] Discontinuation of the product caused normalization of liver enzymes.[123] Also, the use of preparations from *Aristolochia sp*., *Ephedra sinica*, and *Glycyrrhyza glabra*, have been associated with nephrolithiasis, obstructive nephropathy and other features characteristic of chronic renal injury.[121] These are only a few examples that attest to the potential toxic manifestations of products with herbal origin, and therefore the need for cytotoxicity screening in both *in vitro* and *in vivo* settings.

Historically, laboratory animals have been used to assess the risk of chemical entities. However, ethical constraints and legislative changes in many parts of the world have resulted in a gradual shift from the use of animals for preliminary analysis of chemicals and drug molecules.[124] A number of *in vitro* assays have therefore been approved for use in determining the cytotoxic potential of chemicals and other substances meant for human consumption. When doing so, it is important to take cognisance of potential interfere of the constituents with assay substrates. Such interference was observed with the resazurin conversion assay in an experiment aimed at comparing results from four commonly used cytotoxicity assays.[125]

Assessment of chemical substances for cytotoxic effects in mammalian cells enables identification of the concentration that is toxic to half of the population of cells being tested. This concentration is known as the half maximal concentration (IC_{50}).[126] The IC_{50} enables the determination of the possible range of concentrations for further biological activity assessments such as anticancer, antioxidant, antimicrobial, or effect on wounds. Cytotoxicity assessment at the early stages allows for the elimination of harmful entities at an early stage in the drug development process, thereby reducing cost and time invested in a product that would not make it to market.[127] In lieu of the legal and ethical constrains associated with use of whole animals for research purposes, cytotoxicity determinations are primarily conducted using cells derived from humans and other mammalian species. In this study, SC-1 fibroblasts, C2C12 myoblasts, and THP-1 differentiated macrophages were purchased from commercial suppliers. These cells were chosen because of their significance in the wound healing process, as discussed in chapter one.[13]

3.1.2. *In vitro* methods for assessing cytotoxicity

Methods available for the *in vitro* determination of cytotoxicity include the measurement of key cellular processes and components such as deoxyribonucleic acid (DNA) synthesis, metabolic activity, adenosine triphosphate (ATP), proliferation markers, and protein content, as well as morphological analysis using microscopy. The first three are monumental components of the cellular proliferation process, whilst the fourth is consequential to the process.

3.1.3. Cytotoxicity assays based on cellular activity

3.1.3.1. Measurement of DNA synthesis

Measurement of DNA synthesis provides an accurate estimation of the number of viable cells. One of the models commonly used in cell research is the incorporation of the radioactive nucleoside ^{3}H-thymidine into DNA of proliferating cells a few hours before the end of the incubation period, followed by extraction of DNA in ice cold trichloroacetic acid (TCA).[128] The extracted material is collected on membrane filters and radiological activity measured using a liquid scintillating counter.[128] Although this model has been used for many years, a limitation is the logistical problems in handling radiolabelled substances.[129] This, in addition to other limitations, such as the cost involved and the lengthy process of developing autoradiographs, has led to the development of alternative DNA synthesis assays which are devoid of radioisotopes.

Several non-radiological nucleoside analogues such as 5-bromo-2'-deoxyuridine (BrdU) are now available for incorporation into proliferating cells for uptake into newly-formed DNA, making direct assessment of cellular proliferation possible without the need for radiolabelling.[130] Cells are exposed to BrdU in a similar way as the thymidine incorporation assay, and presence of the nucleoside analogue in proliferating cells evaluated using immunohistochemistry, immunocytochemistry, enzyme-linked immunosorbent assay (ELISA) or flow cytometry.[130]

3.1.3.2. Measurement of metabolic activity

Viability of cells can also be assessed indirectly by measuring mitochondrial activity. As the powerhouse of the cell, the mitochondrion is responsible for the cell's metabolic activity. Therefore, measurement of the cell's metabolic activity gives an indication of its viability, and by extension helps to estimate the extent of proliferation. This is achieved by using compounds such as Alamar blue (resazurin) and tetrazolium salts, where reduction occurs in metabolically-active cells due to the activity of dehydrogenase enzymes, which yields resorufin and formazan dye, respectively, with a corresponding change in colour that can be measured spectrophotometrically. The tetrazolium salts commonly employed are 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS), 2-(4-iodophenyl)-3-

(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WT1), and the most recent addition, 2,3,5-triphenyl-2H-tetrazolium chloride (TTC).[126, 131-133] Whereas the use of MTT has been limited as a result of its cellular toxicity and insolubility in standard culture medium, the other tetrazolium salts, as well as Alamar blue, are less cytotoxic and much more soluble.[131] The Alamar blue assay is additionally more advantageous with respect to instrumental requirements. Resorufin is a fluorescent compound, hence in addition to spectrophotometry, it can also be detected and measured fluorometrically.[131]

3.1.3.3. ATP measurement

Cellular ATP content has a direct relationship with a cells metabolic activity, hence serves as an indicator of cellular proliferation. One of the most common methods used to measure this parameter is the bioluminescent method. This method is based on the ability of ATP to facilitate the conversion of luciferin to oxyluciferin by the enzyme luciferase.[134] Measurement of the chemiluminescent signal, produced as a result of oxyluciferin formation, with the aid of a luminometer gives a direct estimate of the cells' ATP content.[134]

3.1.3.4. Measurement of proliferation markers

Another indirect method of determining cellular proliferation is to detect antigens of proteins such as Ki-67, topoisomerase IIβ, and proliferating cell nuclear antigen (PCNA), that are only present in proliferating cells. The Ki-67 protein, for instance, is present during all active stages of the cell cycle (G1, S, G2, mitosis), but absent from resting or quiescent cells (G0), making it an ideal marker for determining cell proliferation.[135] Using respective antibodies, the presence of these proteins can be detected with the aid of immunocytochemistry and immunofluorescence techniques.

3.1.3.5. Cellular protein content

The protein content of cells is another parameter that could be assessed as determination of cytotoxicity. An assay introduced in 1990 by Skehan *et al*., the SRB assay, has become one of the most popular cytotoxicity assays because of its ability to measure protein content of viable cells.[136] The assay is based on the ability of the soluble bright pink aminoxanthene SRB dye to bind to cellular protein that has been fixed to culture plates by cold TCA. The protein content is then determined

colorimetrically. Advantages of the assay include increased linearity and sensitivity, compared with other colorimetric assays.[136, 137]

3.2. Aim and objectives of chapter

This chapter was aimed at examining the prepared extracts for possible cytotoxic effects in C2C12 myoblasts, SC-1 fibroblasts, and THP-1 macrophages.

The objectives were to:

- culture the cells using standard culturing conditions.
- differentiate THP-1 monocytes into macrophages.
- determine the effect of the extracts on cellular density using the SRB assay.
- evaluate the effect of the extracts on cell morphology using phase contrast, PlasDIC, and fluorescence microscopy.

3.3. Methodology

3.3.1. Culture, maintenance and seeding of cells

The SC-1 fibroblast (CRL-1404), C2C12 myoblast (CRL 1772) and THP-1 monocyte (TIB-202) cell lines were purchased from the American Type Culture Collection (ATCC). The adherent SC-1 fibroblasts and C2C12 myoblasts were 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 37ºC in a humidified incubator under an ambient pressure air atmosphere containing 5% CO_2. The non-adherent THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, USA) supplemented with L-glutamine (2 mM), HEPES (10 mM), sodium pyruvate (1 mM), glucose (2.5 g/L), 2-mercaptoethanol (50 µM), 10% heat-inactivated FCS, and 1% penicillin/streptomycin at 37ºC in a humidified incubator under an ambient pressure air atmosphere containing 5% CO_2. Once cells were confluent, flasks were rinsed with phosphate-buffered saline (PBS) and adherent cells enzymatically detached with Trypsin/Versene solution for 5 min. Cells were then centrifuged (200 *g*, 5 min) and re-suspended in 1 mL 10% FCS-supplemented DMEM. Cell counting was done using the trypan blue exclusion assay and a haemocytometer.

3.3.2. Differentiation of THP-1 monocytic cells

Before conducting bioassays, the non-adherent THP-1 cells were differentiated into macrophages using phorbol 12-myristate 13- acetate (PMA, Sigma-Aldrich, USA).[138] Cells were seeded at the densities of the respective sections, and exposed to 0.1 µM PMA for 48 h, under similar conditions as described above, to allow for differentiation to take place. Following differentiation into macrophages (characterised by cell adhesion microscopically), PMA-containing media was replaced with fresh media and cells used immediately for the assay.

3.3.3. Cytotoxicity determination

3.3.3.1. Cytotoxicity evaluation with SRB

Extracts were assessed for possible cytotoxicity using a modified SRB assay as described by Vichai and Kirtikara.[137] The assay is based on the binding of the SRB dye to basic amino acids of cellular proteins, and colorimetric evaluation provides an estimate of cellular protein content, considered directly proportional to the cell density.[139] Cells (100 µL, 1×10^4 cells/well) were seeded into 96-well plates in 10% FCS-supplemented medium and incubated for a day. To determine the safety profiles of each sample, 100 µL of the extract in culture medium were added to the plates at a final working concentration range of 1-100 µg/mL and incubated for 24 h or 48 h. Serial dilutions of 1% saponin in culture medium served as positive control, whilst 100 µL of DMEM was added as negative control. A 1% (v/v) DMSO in culture medium was used as vehicle control, whilst culture medium only served as the blank control. Due to the low yield of the hexane extract, DMSO concentrations were higher than anticipated. As such, vehicle controls were used to account for any potential cytotoxic effects. It is known that DMSO is cytotoxic, but may be tolerable up to 10% in certain cell cultures.[140]

After the exposure period, cells were fixed with 50 µL of cold TCA solution (50% w/v), and the plate incubated at 4ºC overnight to allow for fixation. After fixation, the plate was gently washed with water (four times) to remove excess TCA and dried in a low-temperature oven (40ºC) for 60 min. The fixed cells were stained with 100 µL of 0.057% (w/v in 1% acetic acid) SRB solution and the plate incubated for 30 min at room temperature. Dried plates were washed twice with 200 µL of 1% acetic acid solution (v/v) to remove excess unbound dye, and the plate dried again. The bound

dye was dissociated using 200 µL of a 10 mM Tris-base solution (pH 10.5) amidst shaking for 30 min on an electronic shaking device. Absorbance was then measured at 540 nm with a reference wavelength of 630 nm,[141] using a plate reader (BioTek ELx800) and cell density calculated as follows:

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

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

3.3.3.2. Morphological observation

3.3.3.2.1. Light microscopy

Cells were cultured in 24-well plates at a density of 2.5×10^4 cells/well in 400 µL media, and treated in a similar way as described in Section 3.3.3.1, except cells were only treated with 10 and 100 µg/mL extract. Following incubation, cells were visualised with a phase contrast microscope (Zeiss, Oberkochen, Germany) at 10× magnification for signs of apoptosis and necrosis. Apoptotic cells are characterized by cytoplasmic shrinkage, nuclear condensation, membrane blebbing and apoptotic body formation.[142] Necrosis is characterized by swelling and cell lysis.[142] PlasDIC was also conducted at 40× magnification, and pictures taken and edited using AxioVision 4.

3.3.3.2.2. Live-dead staining

The staining solution consisted of a 1:5 mixture of 2 µg/mL fluorescein diacetate (FDA, Sigma-Aldrich, USA) and 10 µg/mL propidium iodide (PI, Sigma-Aldrich, USA) in PBS, respectively. Cells were seeded and exposed to extracts as described in Section 3.3.3.2.1. Following incubation, cells were washed twice with PBS (200 µL) and stained for 5 min with 50 µL of the FDA/PI staining solution in the dark. Cells were washed twice with 200 µL PBS, and covered with FCS-free DMEM (200 µL) for evaluation using a fluorescence microscope (Axiovert 40 CFL) with an inverted camera (ZEISS AxioCam MRm). All images were captured at 10× magnification.

3.4. Statistical analysis

Data represents results of at least three independent experiments conducted in technical triplicates. Statistical analysis was performed using Graphpad Prism 7 data analysis software. Data was expressed as the mean ± SEM. Non-linear regression was used to determine IC_{50}. The difference between groups was determined by a Kruskal-Wallis test followed by Dunn's post-hoc test. *p* values < 0.05 were considered significant.

3.5. Results and discussion

Due to possible interferences with compounds, the SRB assay was used in conjunction with microscopy for visual confirmation.[125, 137] A crude extract is generally considered to display *in vitro* cytotoxicity if the IC_{50} obtained after treatment is <20 µg/mL.[143] Cellular density was decreased by more than 95% at both treatment periods upon treatment of fibroblasts and myoblasts with 1% saponin, the positive control. This chemical, however, only caused a maximum of 78% reduction in the density of the differentiated macrophages. Cell density in the vehicle control cells was largely unaltered after both treatment periods.

3.5.1. *Aspilia africana*

All extracts of *A. africana* displayed IC_{50}'s greater than the maximum concentration tested (100 µg/mL) after exposure for 24 h and 48 h (Figure 20). The methanol and ethnomedicinal extracts were the most cytotoxic to C2C12 myoblasts and SC-1 fibroblasts, respectively. Myoblast cell density, following treatment with the methanol extract, was decreased by 38.8% and 32.0% after 24 h and 48 h exposure, respectively. Similarly, the ethyl acetate extract of the plant decreased myoblast cell density by 31.7% and 30.0%, respectively, after 24 h and 48 h, when compared to the negative controls. The extracts which demonstrated the least cytotoxic potential in C2C12 myoblasts were the hexane, water, and ethnomedicinal extracts. Cell densities, following 24 h and 48 h exposure with these extracts up to a maximum concentration of 100 µg/mL, were decreased by a maximum of 21.0%. Overall, however, the effect on myoblast cell density was less pronounced after 48 h, compared to 24 h. This implies that the cells recuperated after 24 h, or the block exhibited by the extracts on cell growth was only temporary.[143, 144]

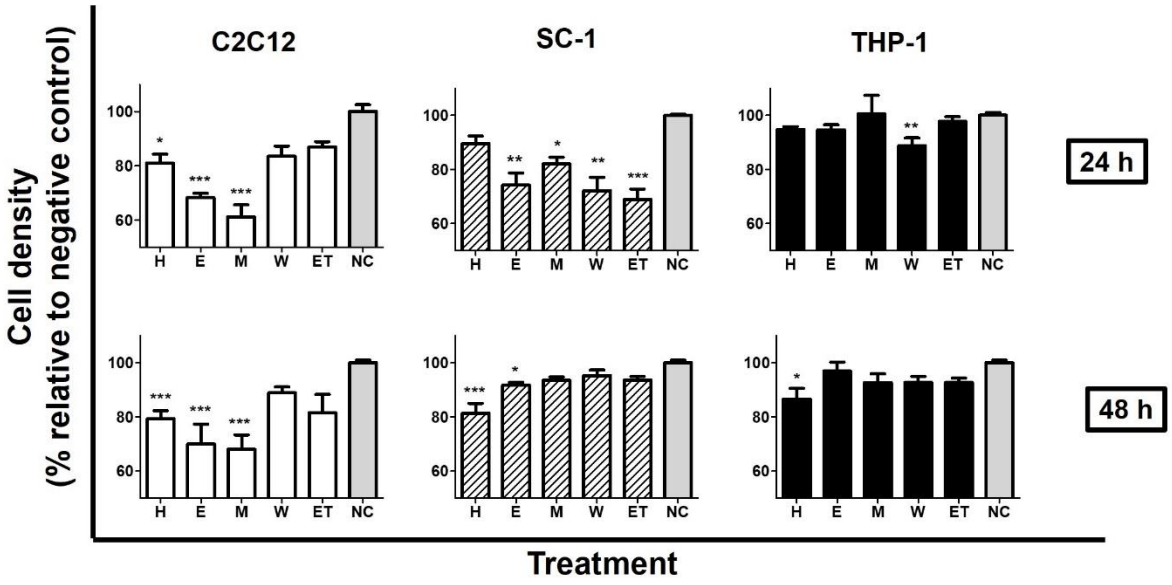

Figure 20: Cell density of C2C12 myoblast, SC-1 fibroblast and THP-1 monocyte cells after 24 h (top) and 48 h (bottom) exposure to 100 µg/mL hexane (H), ethyl acetate (E), methanol (M), water (W), and ethnomedicinal (ET) extracts of *A. africana*. NC, negative control; Statistical significance relative to negative control: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

The ethnomedicinal extract of *A. africana* had the most pronounced effect on the density of SC-1 fibroblasts. The extract caused a 31.1% decrease in cell density after 24 h exposure. The effect of the ethyl acetate and water extracts were less prominent after 24 h, although significantly different from the negative controls ($p < 0.01$). These extracts decreased fibroblast cell density by 25.8% and 27.8%, respectively, after 24 h. Except for the hexane extract, cells treated with the other extracts recuperated after 24 h. Cell density, following 48 h exposure to the hexane extract, was reduced by 18.7%. This suggests possible fibroblast cytotoxicity following long term exposure to higher concentrations of the extract.

The THP-1 macrophages were the least affected by exposure to the *A. africana* extracts. The most cytotoxic extract, hexane, caused a 18.1% reduction in the density of the cells following 48 h exposure to diverse concentrations up to a maximum of 100 µg/mL. Treatment with the other extracts resulted in less than 15% loss in macrophage cell densities at both exposure periods. Generally, the effect of the extracts on the

cells was more prominent after 24 h. Cells treated with the hexane and methanol extracts had lower cell densities after 48 h exposure, indicating a possible long-term effect on macrophages.

Morphological assessment of treated cells was done using phase contrast, PlasDIC, and fluorescence microscopy (Figures 21 – 26). Micrographs of cells treated with 100 µg/mL hexane for 48 h were used to represent data, as no difference was observed in comparison to other treatments. Observation of all treated cells with the inverted phase contrast microscope showed that they had similar morphology as the negative controls. Typical morphological features of cytotoxicity such as plasma membrane blebbing, cell vacuolisation, echinoid spiking, chromatin condensation, formation of apoptotic bodies, cell shrinkage, and nuclear fragmentation were absent in extract-treated cells (Figures 21A, 23A, 25A). In contrast, cells treated with the positive control, saponin, were morphologically different, showing clear signs of cytotoxicity such as absence of noticeable cell membranes, decreased cell density, and cellular debris. A similar outcome was observed at 40x magnification using PlasDIC (Figures 21B, 23B, 25B).

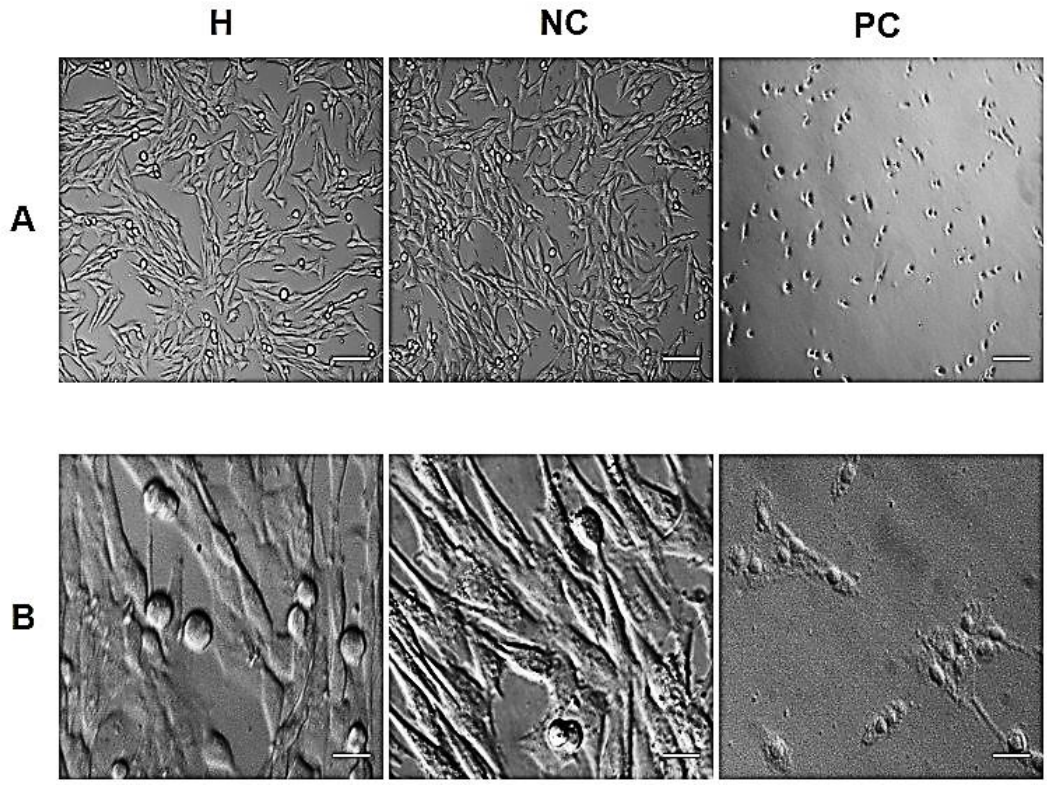

Figure 21: Phase contrast (**A**) and PlasDIC (**B**) images of C2C12 myoblasts exposed to 100 µg/mL of *A. africana* extracts. H, hexane extract; NC, negative control; PC,

positive control. Images were captured at 10× (**A**) and 40× magnification (**B**); scale bars: 100 µm (**A**) and 25 µm (**B**).

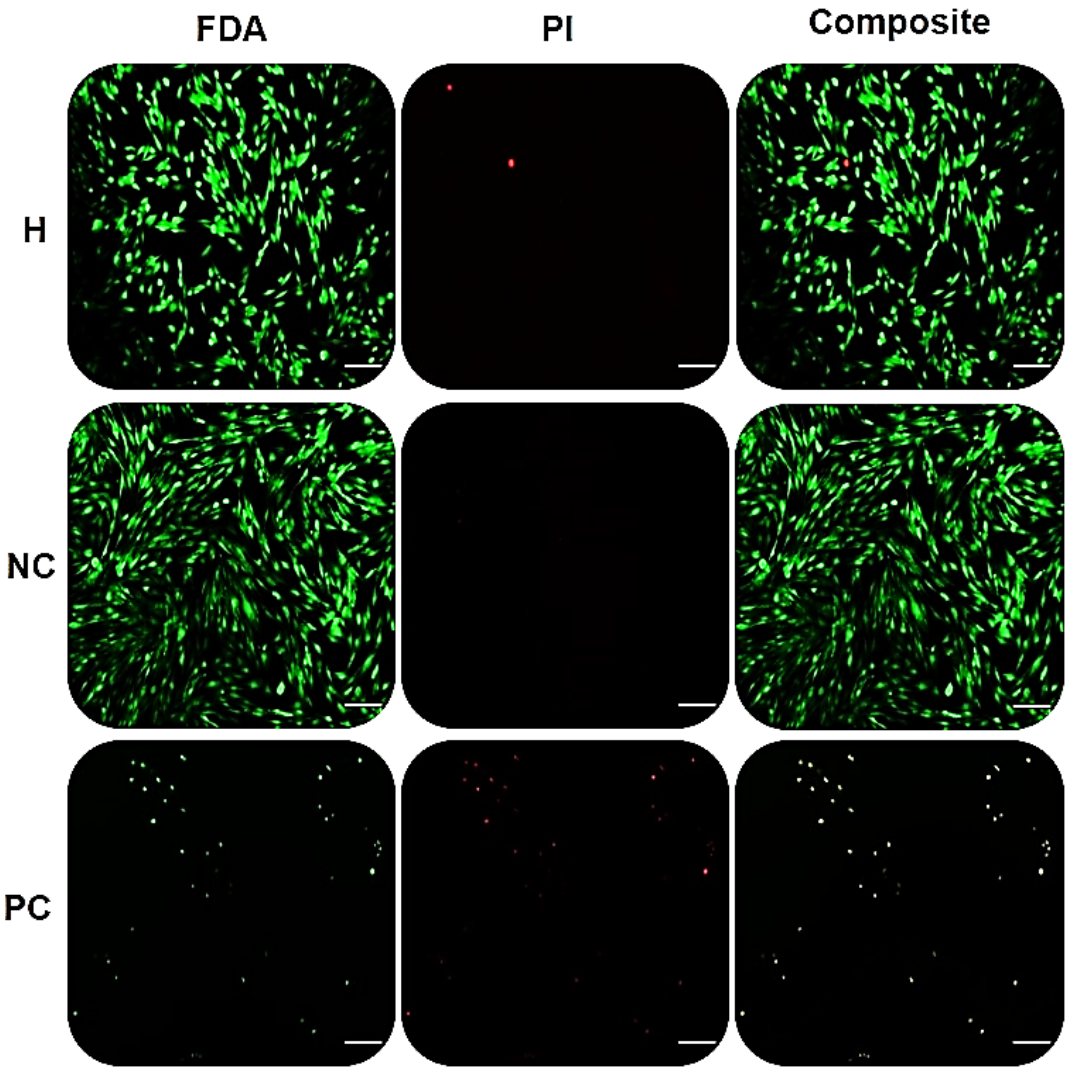

Figure 22: Evaluation of the cytotoxicity of *A. africana* extracts in C2C12 myoblasts using live-dead staining. H, hexane extract; NC, negative control; PC, positive control; FDA, fluorescein diacetate; PI, propidium iodide. 10x magnification; scale bar: 100 µm.

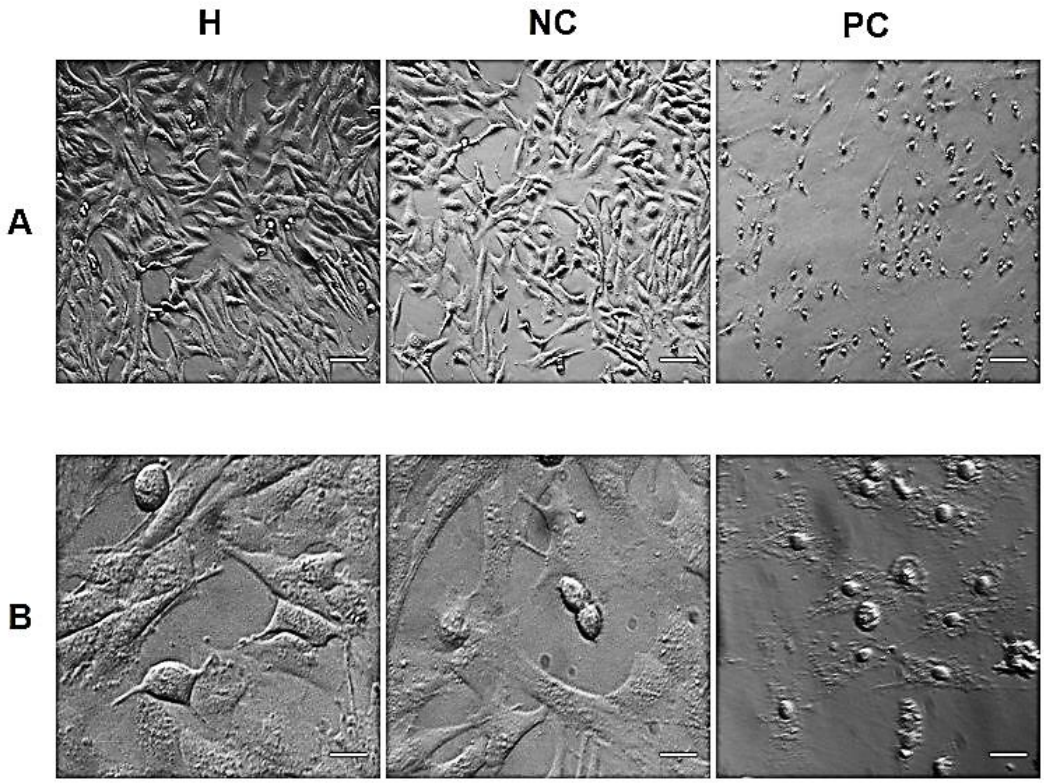

Figure 23: Phase contrast (**A**) and PlasDIC (**B**) images of SC-1 fibroblasts exposed to 100 µg/mL of *A. africana* extracts. H, hexane extract; NC, negative control; PC, positive control. Images were captured at 10× (**A**) and 40× magnification (**B**); scale bars: 100 µm (**A**) and 25 µm (**B**).

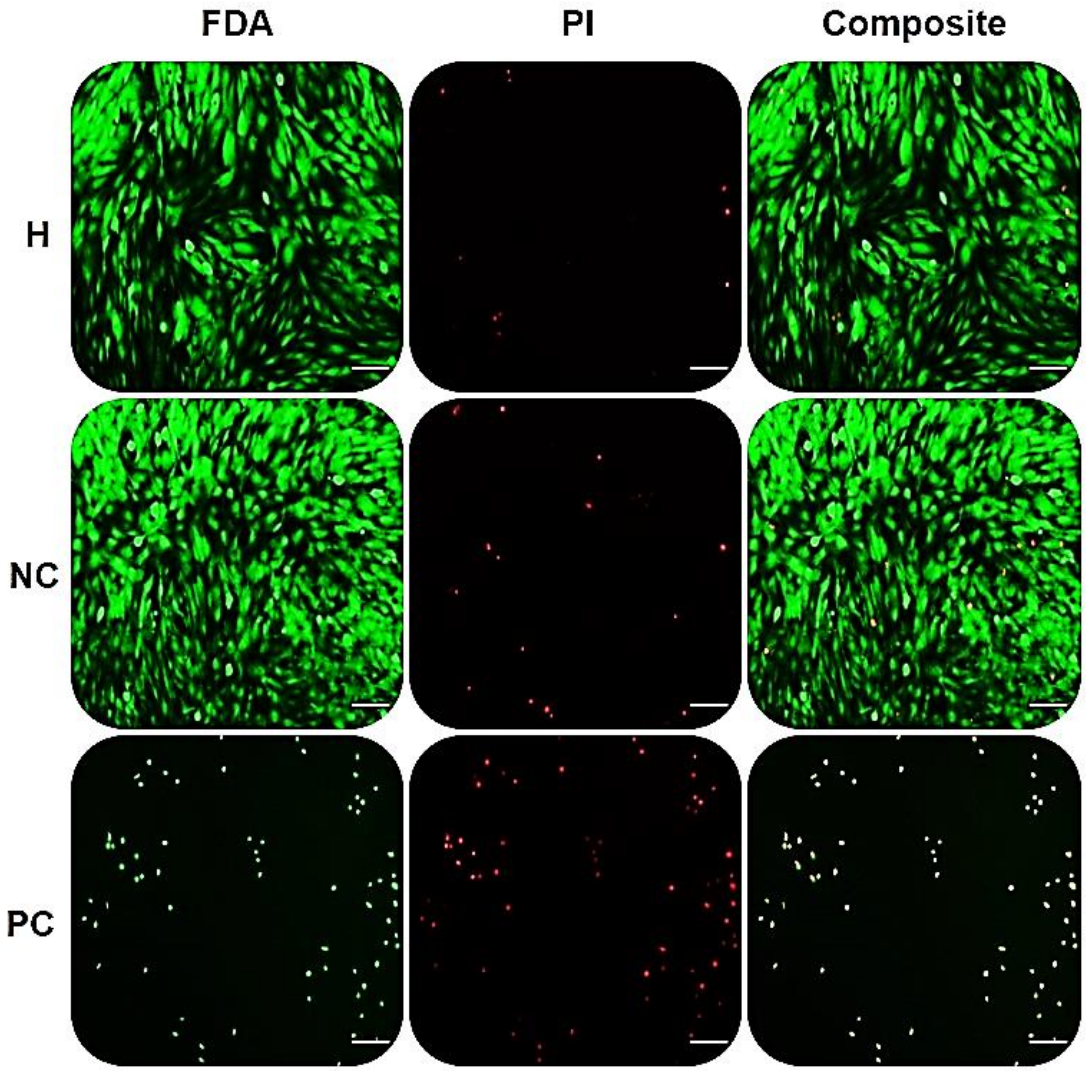

Figure 24: Evaluation of the cytotoxicity of *A. africana* extracts in SC-1 fibroblasts using live-dead staining. H, hexane extract; NC, negative control; PC, positive control; FDA, fluorescein diacetate; PI, propidium iodide. 10× magnification; scale bar: 100 µm.

Staining cells with the fluorescent dyes FDA and PI, and observing under a fluorescence microscope, showed no difference between treated and untreated cells, though some treated cells had marginally lower cell density (Figures 22, 24, 26). This correlates with findings from the SRB assay and the other microscopic evaluations. Green fluorescence represents viable cells stained with FDA, whilst the red represents PI staining of dead cells or cells with a compromised membrane. The composite is a combination of PI and FDA staining. Cells treated with the saponin positive control

were devoid of noticeable membranes, an indication of the cytotoxic potential of saponin.

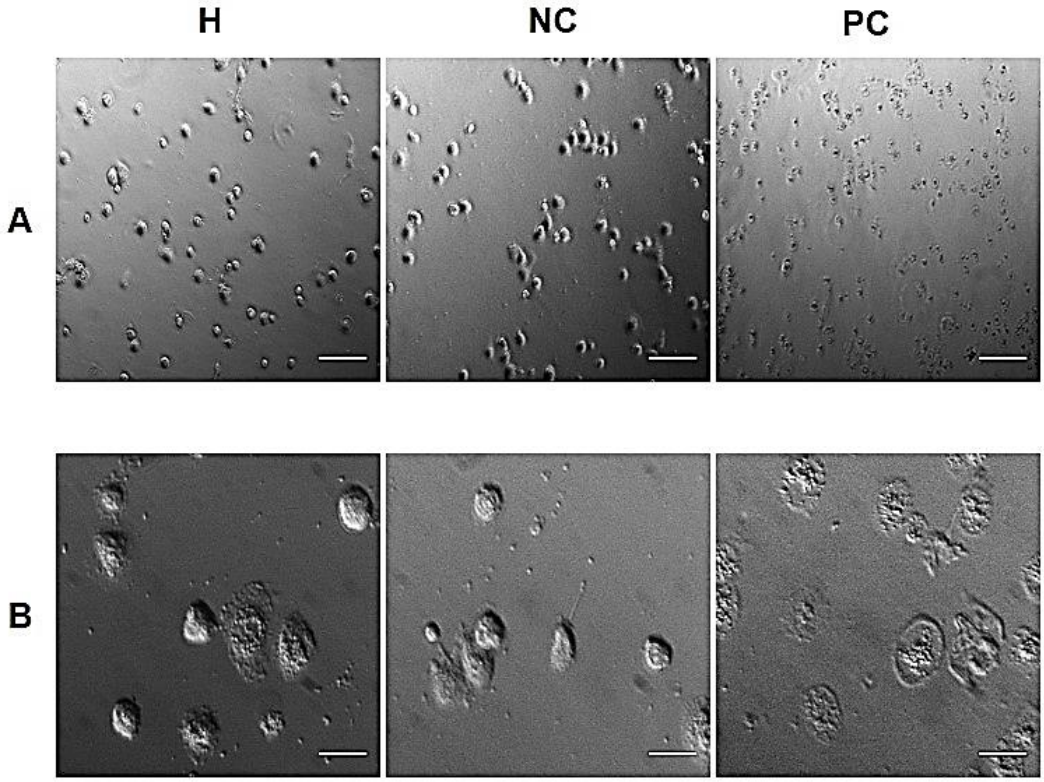

Figure 25: Phase contrast (**A**) and PlasDIC (**B**) images of THP-1 macrophages exposed to 100 µg/mL of *A. africana* extracts. H, hexane extract; NC, negative control; PC, positive control. Images were captured at 10× (**A**) and 40× magnification (**B**); scale bars: 100 µm (**A**) and 25 µm (**B**).

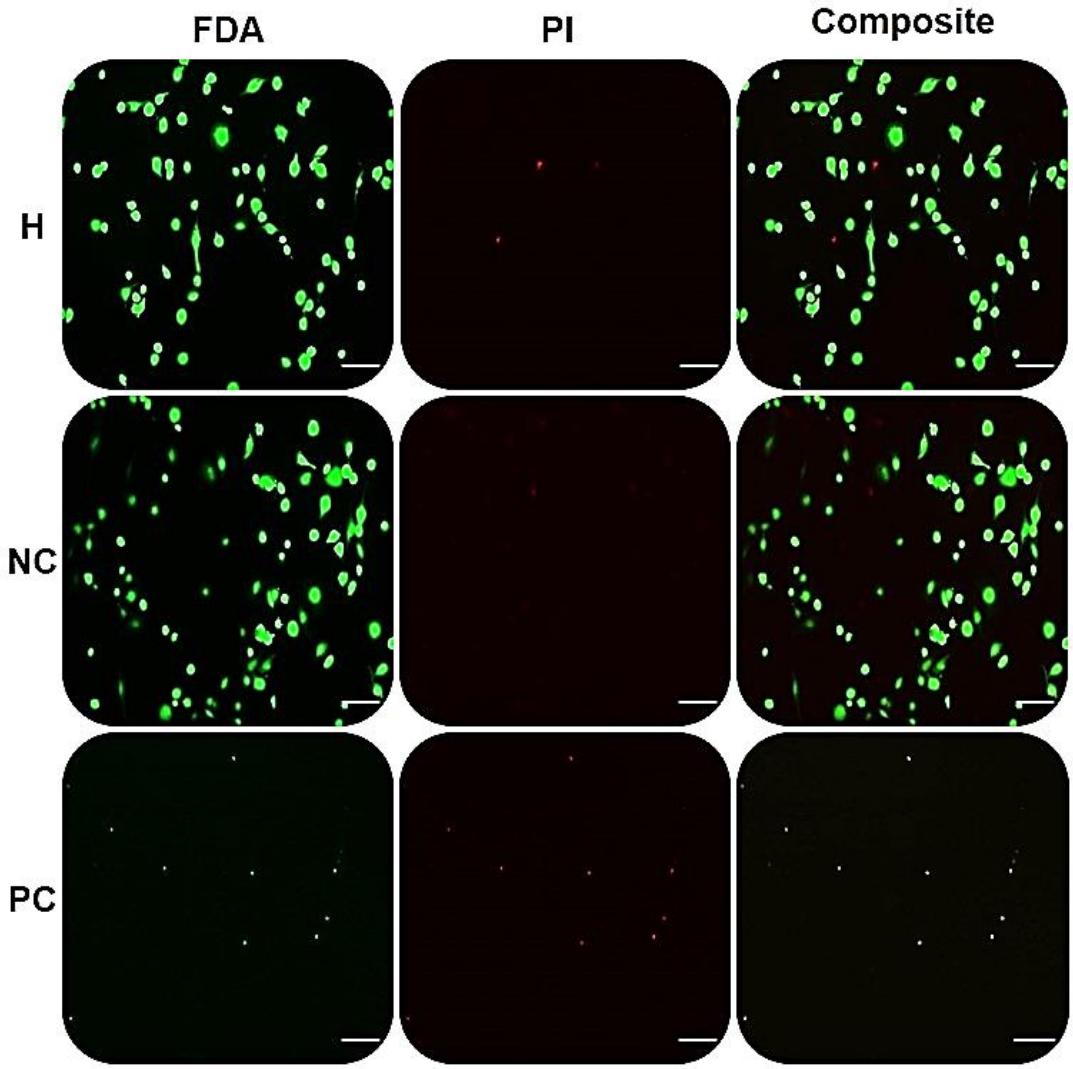

Figure 26: Evaluation of the cytotoxicity of *A. africana* extracts in THP-1 macrophages using live-dead staining. H, hexane extract; NC, negative control; PC, positive control; FDA, fluorescein diacetate; PI, propidium iodide. 10x magnification; scale bar: 100 µm.

Only a few publications have given an account of the cytotoxic effects of *A. africana*, most being *in vivo* in nature. Froelich and colleagues reported that the petroleum ether, ethyl acetate, and methanol extracts displayed minimal cytotoxicity (IC_{50} > 50 µg/mL) in the human hepatocellular carcinoma (HepG2) and urinary bladder carcinoma (ECV-304) cells.[145] Other *in vivo* studies, including that of Okoli *et al*. (2007), have also reported minimal or no toxicity in rodents.[74]

Therefore, it appears that this is the first study to report the cytotoxic potential of *A. africana* extracts in fibroblasts, myoblasts, and macrophages. Neither of the two marker compounds detected in the plant, ascorbic acid and quercetin, are known to

be cytotoxic to normal human cells, though the latter reportedly inhibits some cancer cells.[146] Therefore, it is not surprising that the plant exhibited minimal cytotoxicity in the mammalian cells evaluated. It is however possible that the extracts could potentially contain some cytotoxic alkaloids, phenols, or other groups of compounds that may be responsible for the minimal cytotoxicity observed.

3.5.2. *Boerhavia diffusa*

All *B. diffusa* extracts produced minimal or no cytotoxicity (IC_{50} > 100 µg/mL) in the three cell lines tested over a 48 h period (Figure 27). The hexane, ethyl acetate, and water extracts were the most cytotoxic in C2C12 cells. The ethyl acetate and water extracts decreased myoblast cell density by 20.8% and 19.3%, respectively, 24 h exposure. Although the hexane extract did not affect cell density after 24 h exposure, a significant difference ($p < 0.05$) in myoblast density was observed after 48 h. Cell density, following treatment with all other extracts, was comparable to the negative control. The marginal effect of the hexane extract on myoblasts after 48 h exposure suggests a possible long term cytotoxic effect upon treatment with higher concentrations. Although the ethnomedicinal extract of the plant also decreased cell density by 16% after 24 h, growth of treated cells was observed to be similar to the negative controls after 48 h.

Apart from the hexane extract of *B. diffusa*, none of the other extracts of the plant significantly affected SC-1 fibroblast cell density over a period of 48 h exposure. This extract decreased fibroblast density by 15.9% and 15.1%, following exposure for 24 h and 48 h, respectively. On the contrary, THP-1 macrophage density was not affected by exposure to the *B. diffusa* extracts. The marginal cytotoxic effect of the hexane extract in myoblast and fibroblast cells could be a result of the presence of some cytotoxic alkaloids, flavonoids, or phenols, major phytochemical groups detected in the extract (Table 7). These phytoconstituents are known to contain compounds with cytotoxic potential.[78]

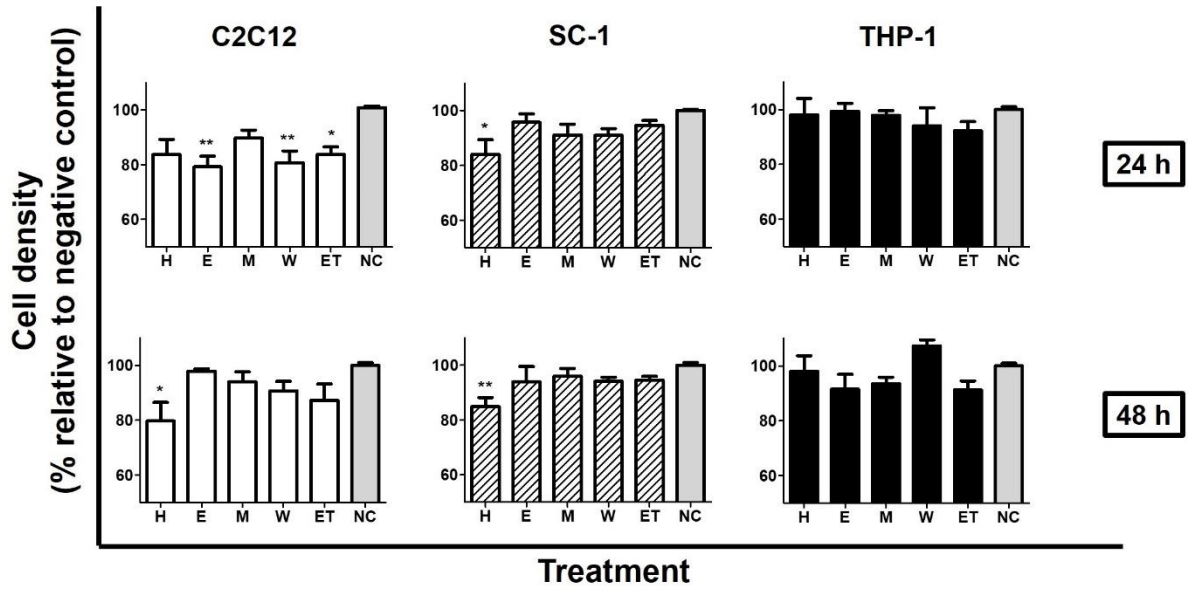

Figure 27: Cell density of C2C12 myoblast, SC-1 fibroblast and THP-1 monocyte cells after 24 h (top) and 48 h (bottom) treatment with 100 µg/mL of the hexane (H), ethylacetate (E), methanol (M), water (W), and ethnomedicinal (ET) extracts of *Boerhavia diffusa*. NC, negative control. Statistical significance compared to the negative control: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Morphological evaluation of the cells confirmed that exposure to the *B. diffusa* extracts did not produce prominent cytotoxic effects in the cells. Phase contrast (Figures 28A, 30A, 32A) and PlasDIC (Figures 28B, 30B, 32B) did not reveal any sign of cytotoxicity following exposure to the extracts. All extract-treated cells had a similar morphological appearance to the negative control. No pronounced cell death or damage was observed. Moreover, cellular assessment by live-dead staining (Figures 29, 31, 33) did not show conspicuous difference in cell density between treated and untreated cells.

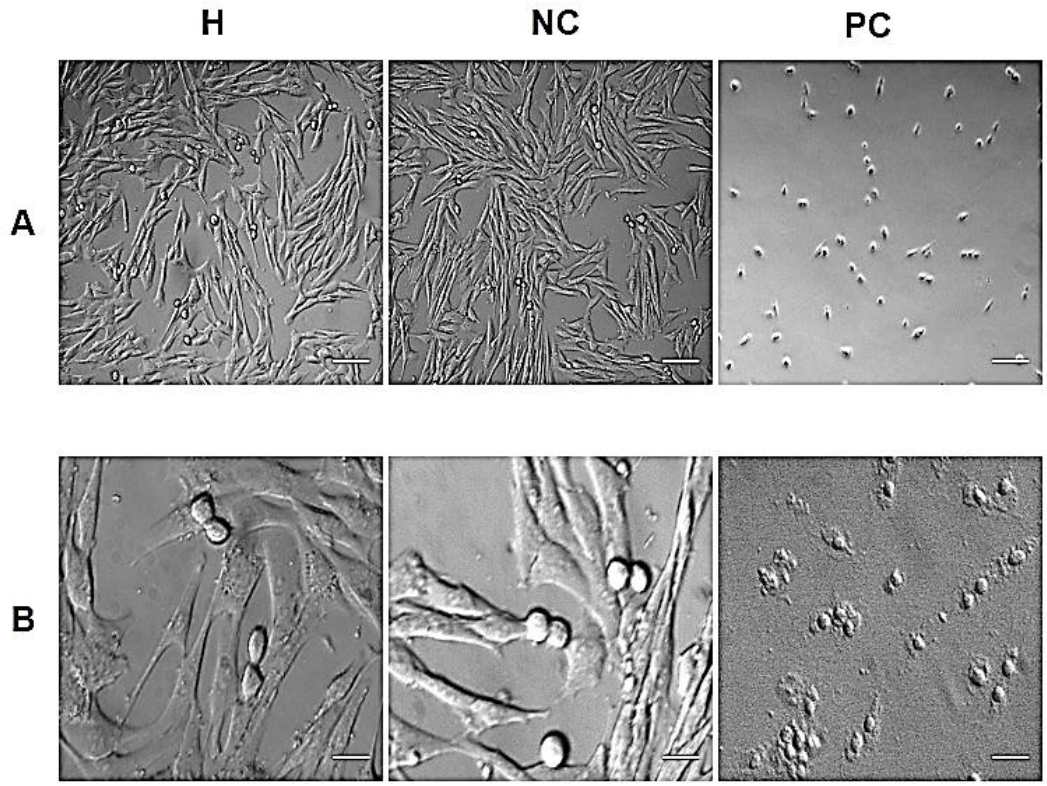

Figure 28: Phase contrast (**A**) and PlasDIC (**B**) images of C2C12 myoblasts exposed to 100 µg/mL of *B. diffusa* extracts. H, hexane extract; NC, negative control; PC, positive control. Images were captured at 10× (**A**) and 40× magnification (**B**); scale bars: 100 µm (**A**) and 25 µm (**B**).

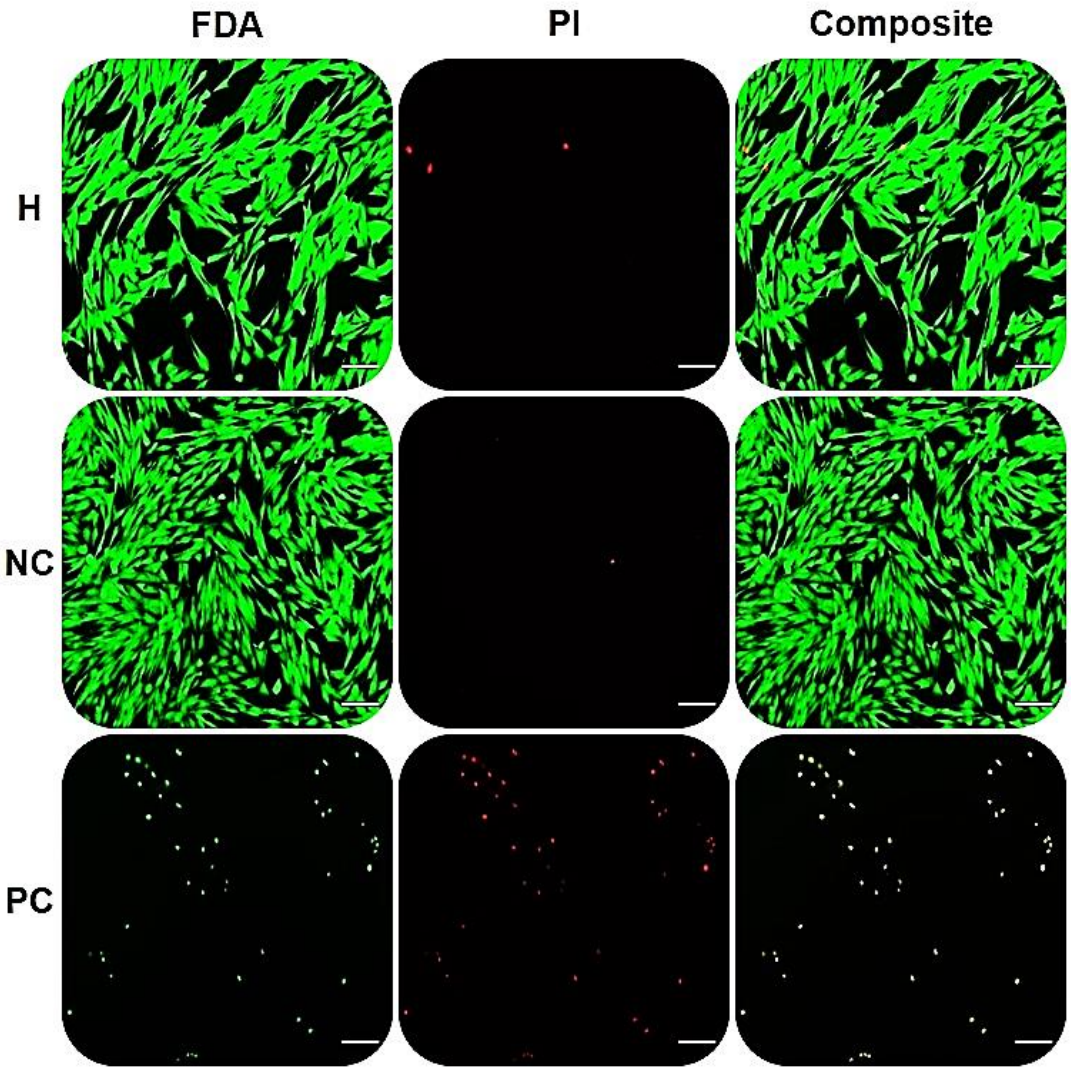

Figure 29: Evaluation of the cytotoxicity of *B. diffusa* extracts in C2C12 myoblasts using live-dead staining. H, hexane extract; NC, negative control; PC, positive control; FDA, fluorescein diacetate; PI, propidium iodide. 10x magnification; scale bar: 100 µm.

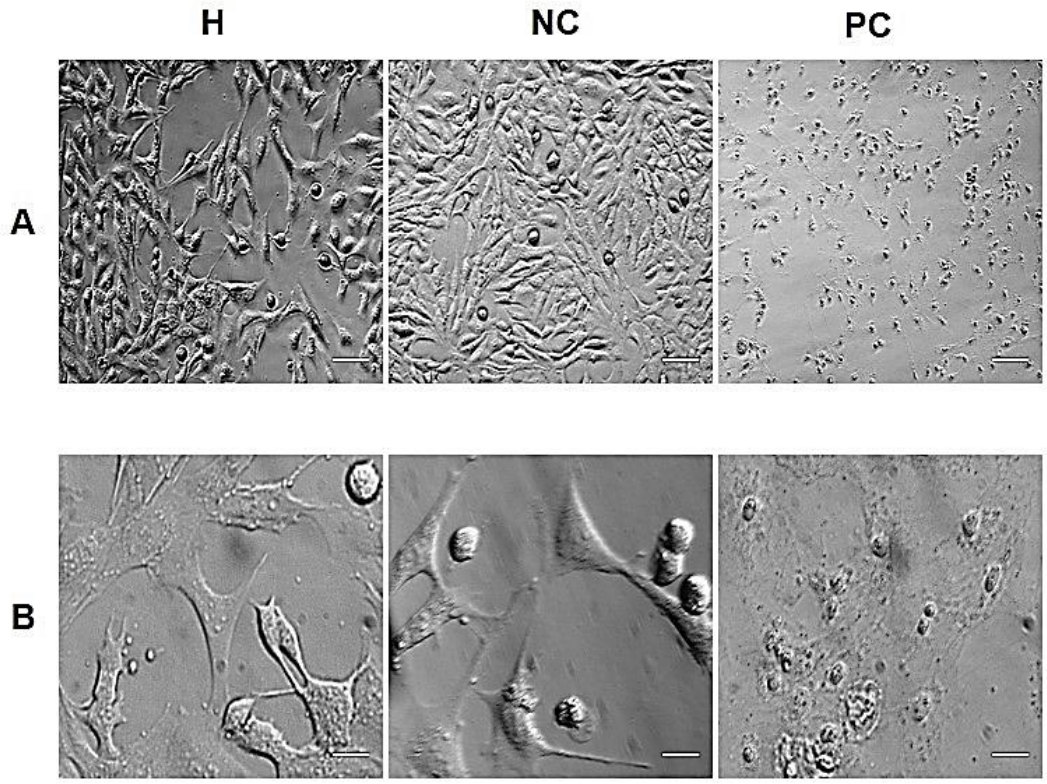

Figure 30: Phase contrast (**A**) and PlasDIC (**B**) images of SC-1 fibroblasts exposed to 100 µg/mL of *B. diffusa* extracts. H, hexane extract; NC, negative control; PC, positive control. Images were captured at 10× (**A**) and 40× magnification (**B**); scale bars: 100 µm (**A**) and 25 µm (**B**).

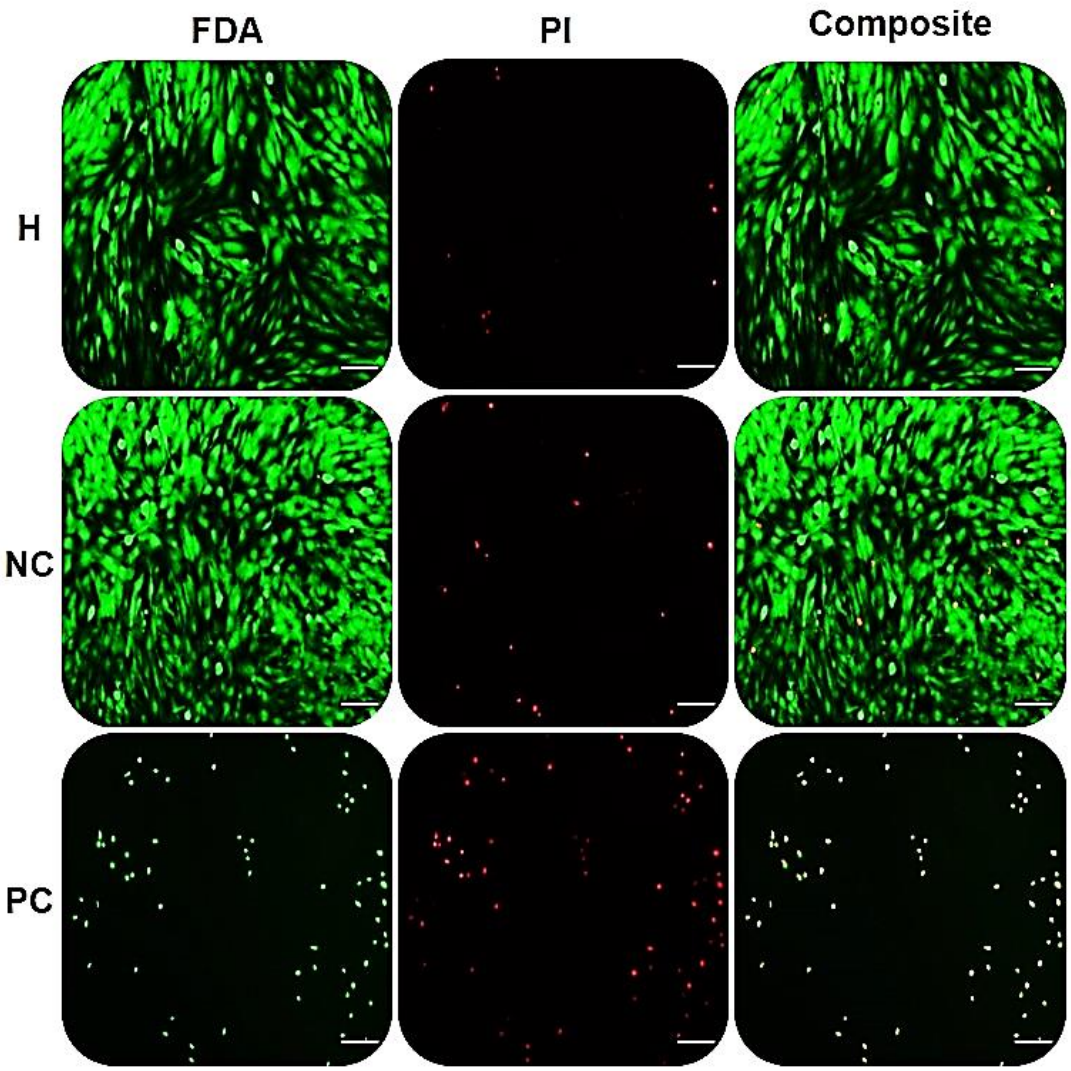

Figure 31: Evaluation of the cytotoxicity of *B. diffusa* extracts in SC-1 fibroblasts using live-dead staining. H, hexane extract; NC, negative control; PC, positive control; FDA, fluorescein diacetate; PI, propidium iodide. 10x magnification; scale bar: 100 µm.

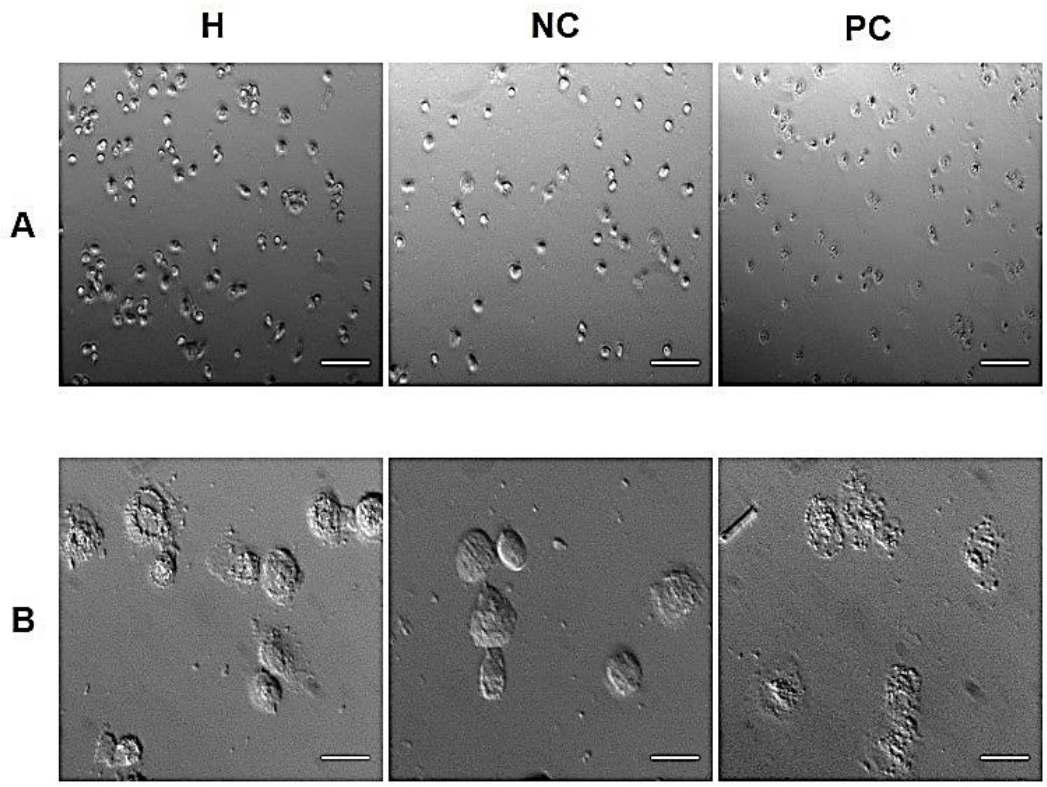

Figure 32: Phase contrast (**A**) and PlasDIC (**B**) images of THP-1 macrophages exposed to 100 µg/mL of *B. diffusa* extracts. H, hexane extract; NC, negative control; PC, positive control. Images were captured at 10× (**A**) and 40× magnification (**B**); scale bars: 100 µm (**A**) and 25 µm (**B**).

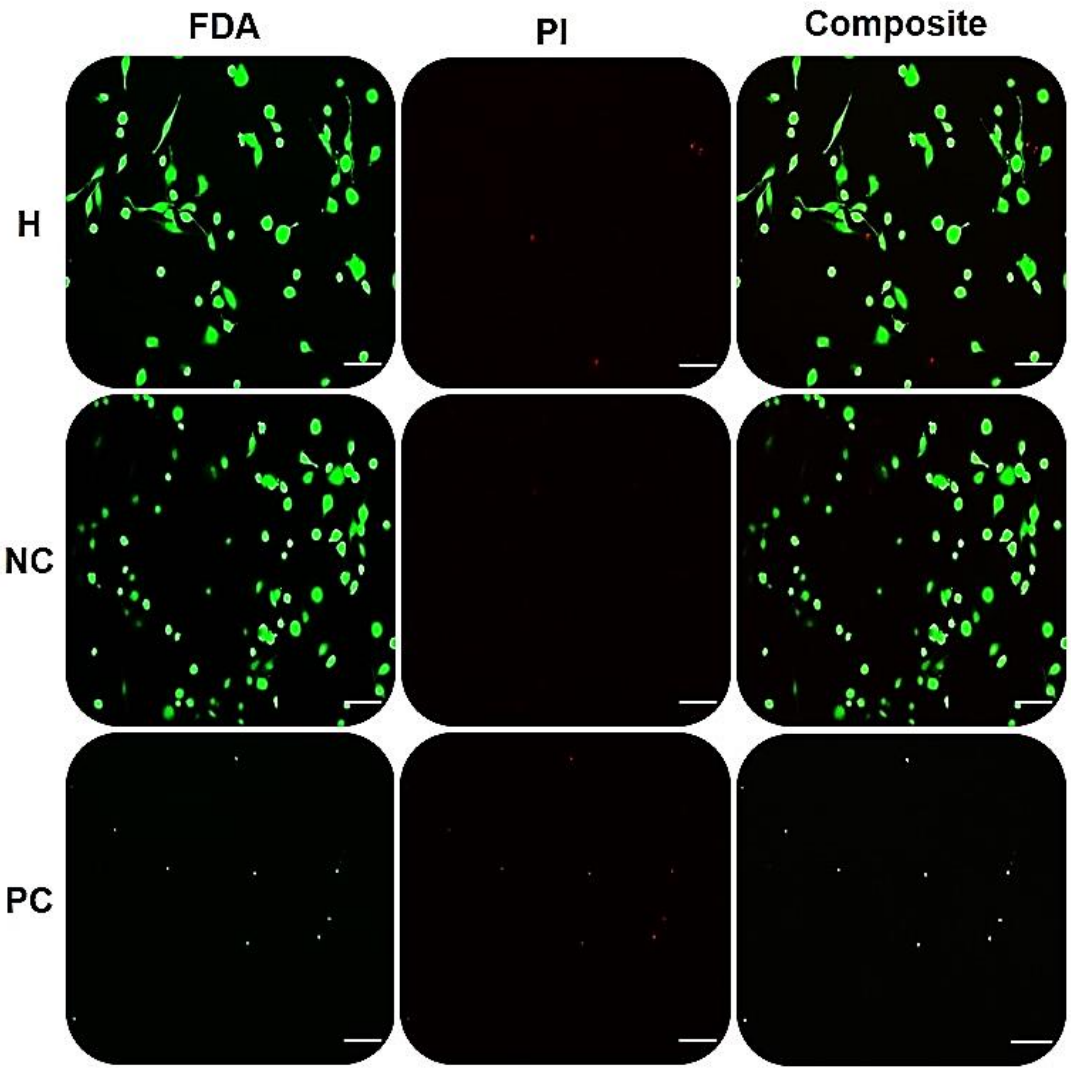

Figure 33: Evaluation of the cytotoxicity of *B. diffusa* extracts in THP-1 macrophages using live-dead staining. H, hexane extract; NC, negative control; PC, positive control; FDA, fluorescein diacetate; PI, propidium iodide. 10x magnification; scale bar: 100 µm

Several reports have detailed the safety of extracts prepared from *B. diffusa* and other species of the *Boerhavia* genus in *in vitro* and animal experiments. Ethanol extracts of *B. diffusa* were found to protect H9C2 cardiomyocytes from arsenic trioxide (ATO)-induced cytotoxicity.[147] Cells, analysed using four separate cytotoxicity assays (MTT, lactate dehydrogenase, neutral red uptake, and phase contrast microscopy), were devoid of cytotoxic manifestations when co-treated with 20 µg/mL of the extract and various doses of ATO up to 7.5 µM.[147] Furthermore, punarnavine, an alkaloid isolated from *B. diffusa* has been reported to have insignificant cytotoxicity in human umbilical vascular endothelial cells (HUVECs).[148] Interestingly, rotenoids isolated from *B.*

erecta showed differential cytotoxicity *in vitro* (Table 9).[149] Boeravinone C, for instance, was cytotoxic to human epithelial carcinoma (HeLa) and human breast cancer (MCF-7) cells, however, it was not cytotoxic to human lung cancer (NCI-H460) cells, suggesting cell-specific cytotoxicity.[149]

Table 9: Differential cytotoxicity of purified compounds from *Boerhavia* species.[149]

Compound	Cell density (%)		
	HeLa	NCI-H460	MCF-7
Boeravinone K	8.86	45.27	69.47
Boeravinone C	10.61	95.54	25.96
10-Demethylboeravinone	15.80	94.85	18.63
Boeravinone L	92.24	92.81	92.00
Boeravinone M	84.31	51.48	14.92
Cucumegastigmane	94.77	97.45	99.54
Boeravinone N	80.15	68.22	64.33
Boeravinone O	92.57	92.73	94.17
Kaempferol 3-O-rutinoside	104.73	96.92	106.15
Camptothecin (positive control)[a]	39.00	22.10	53.00

[a]Tested at concentrations of 0.01 µg/mL for MCF-7 and NCI-H460, and 1 µg/mL for HeLa; purified compounds were tested at 100 µg/mL

In vivo studies have also highlighted the safety and protective effect of extracts prepared from *Boerhavia* spp. Aqueous extracts from *B. erecta* did not exhibit any adverse effect in Wister rats up to a dose of 2 000 mg/kg in an acute and subchronic toxicity study.[150] Also, Aher *et al*. demonstrated in their report that punarnavine, isolated from *Boerhavia* sp., had neither genotoxic nor clastogenic effects in mice.[151]

Findings of the current study therefore add to the published evidence with regards to the safety of *Boerhavia* spp. It is, however, possible that some of the extracts, though shown to have minimal cytotoxicity up to 100 µg/mL in the tested cells, could possess compounds which may be significantly toxic to some cells as demonstrated previously.[149] This could particularly be true for the hexane, ethyl acetate, water, and ethnomedicinal extracts of the plant. These extracts, had no prominent effects on the cell densities of SC-1 (except hexane) and THP-1 cells, but were found to have significant effects on C2C12 cell density in particular when compared to the negative control. Whether or not *B diffusa* contains any cytotoxic compound/s is, however, yet to be determined. Our analysis using UPLC-MS has shown the presence of

kaempferol, quercetin, and rutin in the plant (Figures 16 – 17). Apart from potential anticancer activity,[78] none of these detected compounds is known to exhibit major cytotoxicity in normal cells to the best of the authors knowledge.

3.5.3. *Erythrina senegalensis*

E. senegalensis extracts also displayed slight, but insignificant, cytotoxicity (IC_{50} > 100 µg/mL) in the three cell lines (Figure 34). The methanol and water extracts were the most cytotoxic in C2C12 myoblasts. These extracts reduced myoblast cell density by 15.9% and 22.4%, respectively, after 24 h exposure. All treated cells, however, had comparable cell densities to the negative controls after 48 h exposure. This suggests that the two extracts produced only a temporary block on myoblast growth.

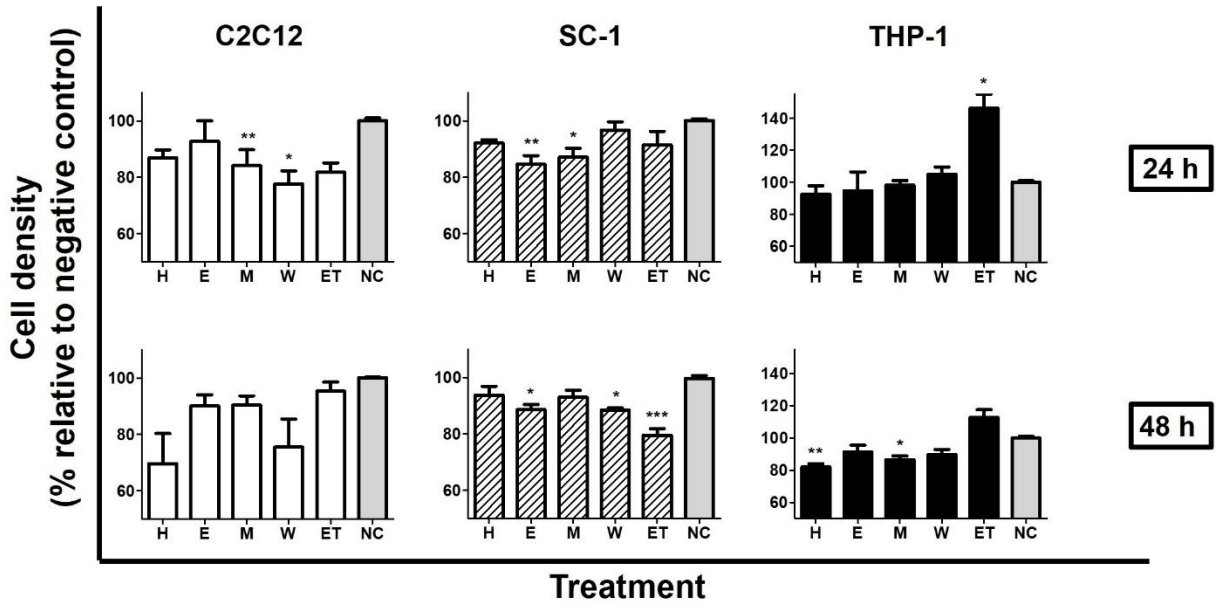

Figure 34: Cell density of C2C12 myoblast, SC-1 fibroblast and THP-1 monocyte cells after 24 h (top) and 48 h (bottom) treatment with 100 µg/mL of the hexane (H), ethyl acetate (E), methanol (M), water (W), and ethnomedicinal (ET) extracts of *Erythrina senegalensis*. NC, negative control; Statistical significance from NC: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

Most of the extracts slightly decreased SC-1 fibroblast cell density. The most prominent effect was observed after 48 h exposure to the ethnomedicinal extract. Fibroblast cell density was decreased by 20.5%, following exposure to the extract, when compared to the untreated cells. Interestingly, the water extract also reduced

cell density by 11.5% after 48 h. Both extracts tested positive for alkaloids, flavonoids, phenols, and terpenes (Table 8). These phytochemical classes possess compounds with known cytotoxic effects.[78] Although no cytotoxic compound/s was detected in these two extracts, the findings suggest a possible cytotoxic effect on fibroblasts upon long term exposure with higher concentrations. The ethyl acetate extract also displayed a similar potential in fibroblasts, although its effect was less pronounced. On the contrary, a temporary block on cell growth was observed with exposure to the methanol extract. Neobavaisoflavone was detected in these extracts (Figures 18 – 19), and is a known cytotoxic isoflavonoid.[114] This compound could be responsible for the marginal cytotoxicity observed in the two extracts.

The THP-1 differentiated macrophages were the least affected by *E. senegalensis* extract exposure. The most cytotoxic extracts were the hexane and methanol extracts, reducing macrophage cell density by 18.1% and 13.5%, respectively, after 48 h. This marginal cytotoxicity could be attributed to the presence of neobavaisoflavone in both extracts (Figures 18 – 19). On the other hand, the ethnomedicinal extract of *E. senegalensis* appears to have temporarily stimulated the proliferation of macrophages. Cell density was 46.2% higher in treated cells compared to untreated cells. This was however comparable in the two groups after 48 h, and suggests the presence of compounds with the ability to promote proliferation of macrophages. Further work is however required to confirm this.

Examination of treated cells for morphological abnormalities and other signs of cytotoxicity also revealed no major difference when compared to the untreated cells. Phase contrast (Figures 35A, 37A, 39A) and PlasDIC (Figures 35B, 37B, 39B) did not reveal any sign of cytotoxicity following exposure to *E. senegalensis*. All extract-treated cells had a similar morphological appearance to the negative control. No pronounced cell death or damage was observed. Moreover, cellular assessment by live-dead staining (Figures 36, 38, 40) did not show conspicuous difference in cell density between treated and untreated cells.

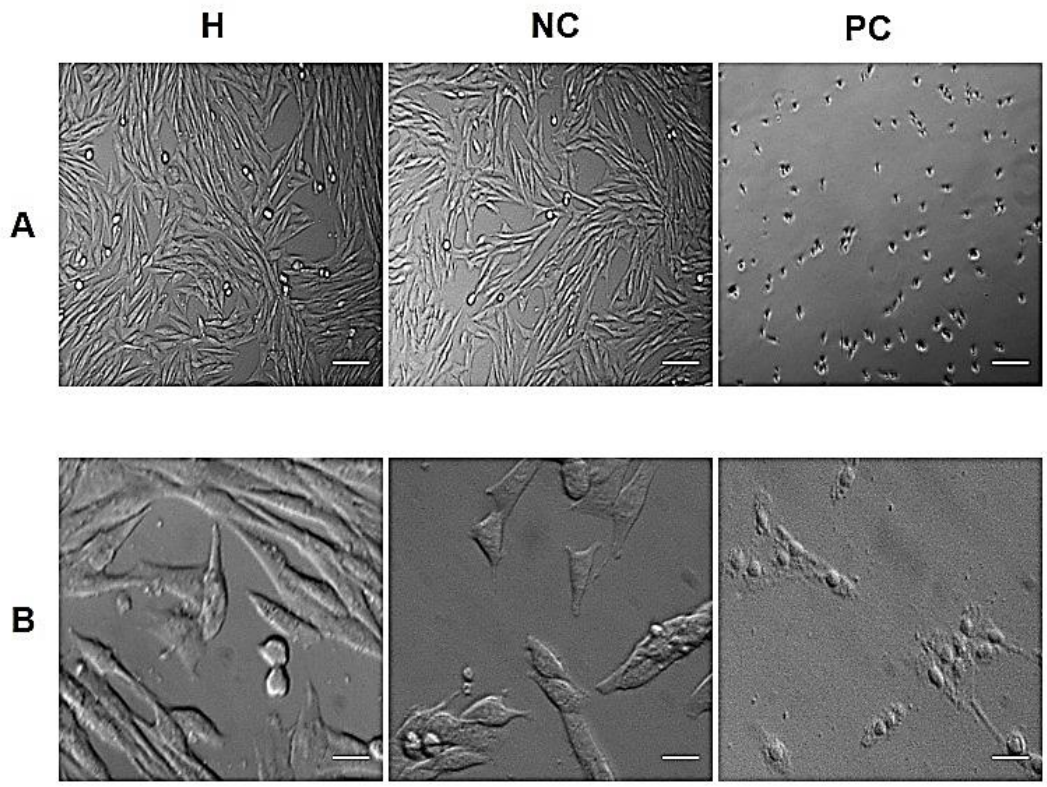

Figure 35: Phase contrast (**A**) and PlasDIC (**B**) images of C2C12 myoblasts exposed to 100 µg/mL of *E. senegalensis* extracts. H, hexane extract; NC, negative control; PC, positive control. Images were captured at 10× (**A**) and 40× magnification (**B**); scale bars: 100 µm (**A**) and 25 µm (**B**).

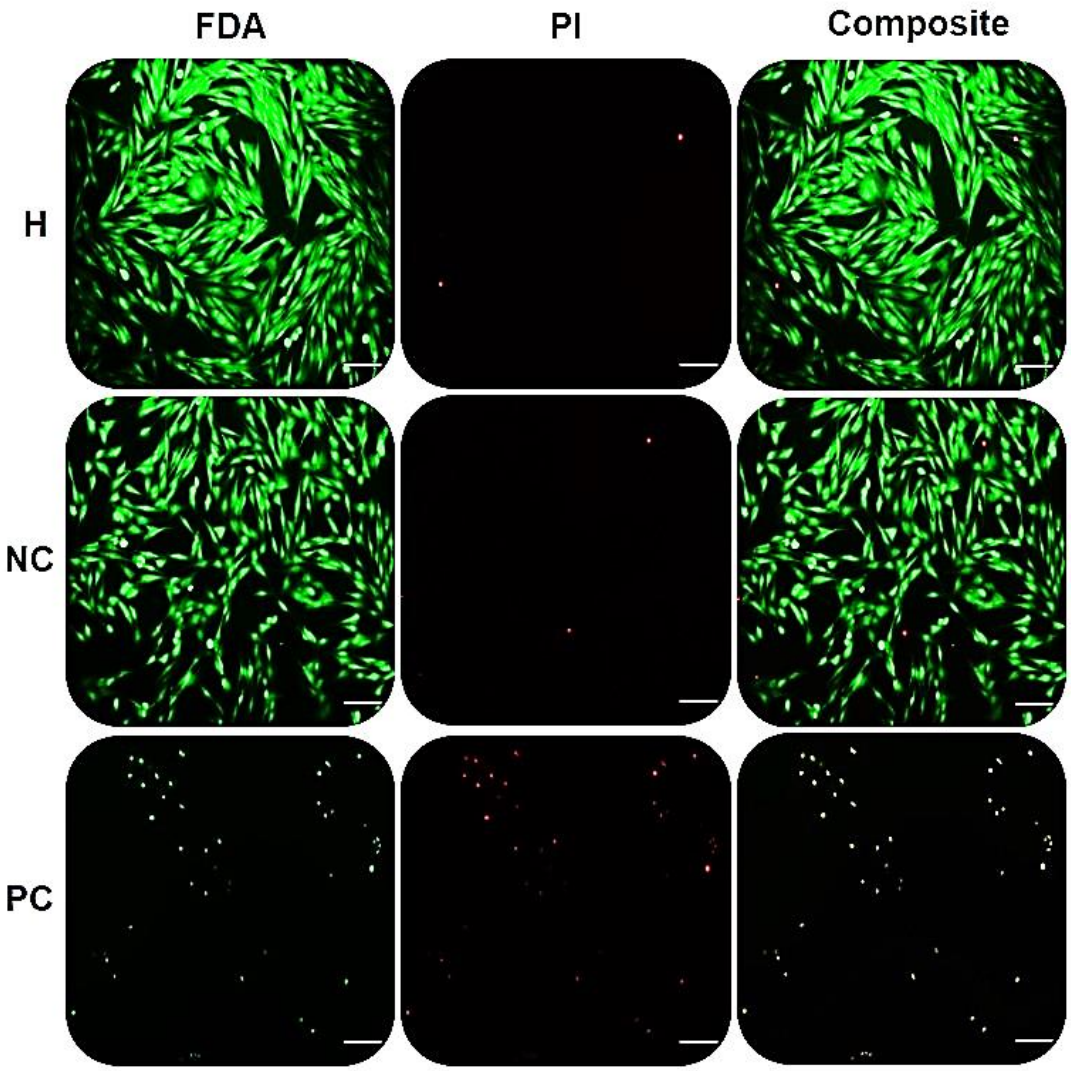

Figure 36: Evaluation of the cytotoxicity of *E. senegalensis* extracts in C2C12 myoblasts using live-dead staining. H, hexane extract; NC, negative control; PC, positive control; FDA, fluorescein diacetate; PI, propidium iodide. 10x magnification; scale bar: 100 µm

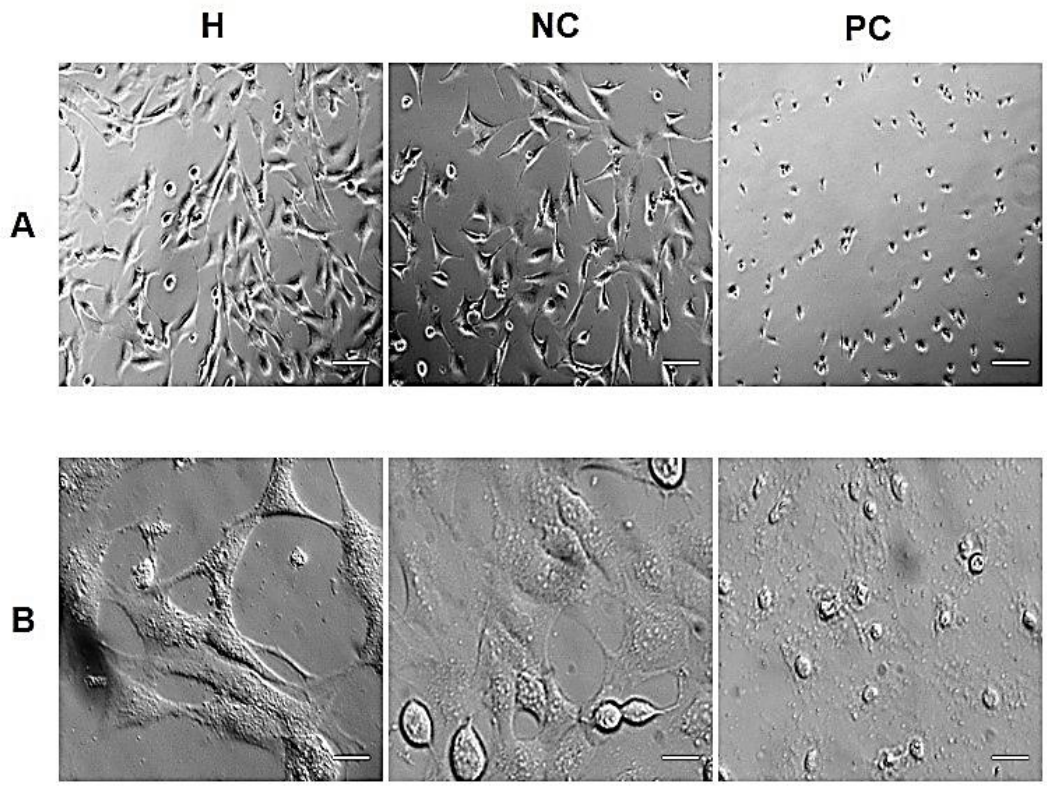

Figure 37: Phase contrast (**A**) and PlasDIC (**B**) images of SC-1 fibroblasts exposed to 100 µg/mL of *E. senegalensis* extracts. H, hexane extract; NC, negative control; PC, positive control. Images were captured at 10× (**A**) and 40× magnification (**B**); scale bars: 100 µm (**A**) and 25 µm (**B**).

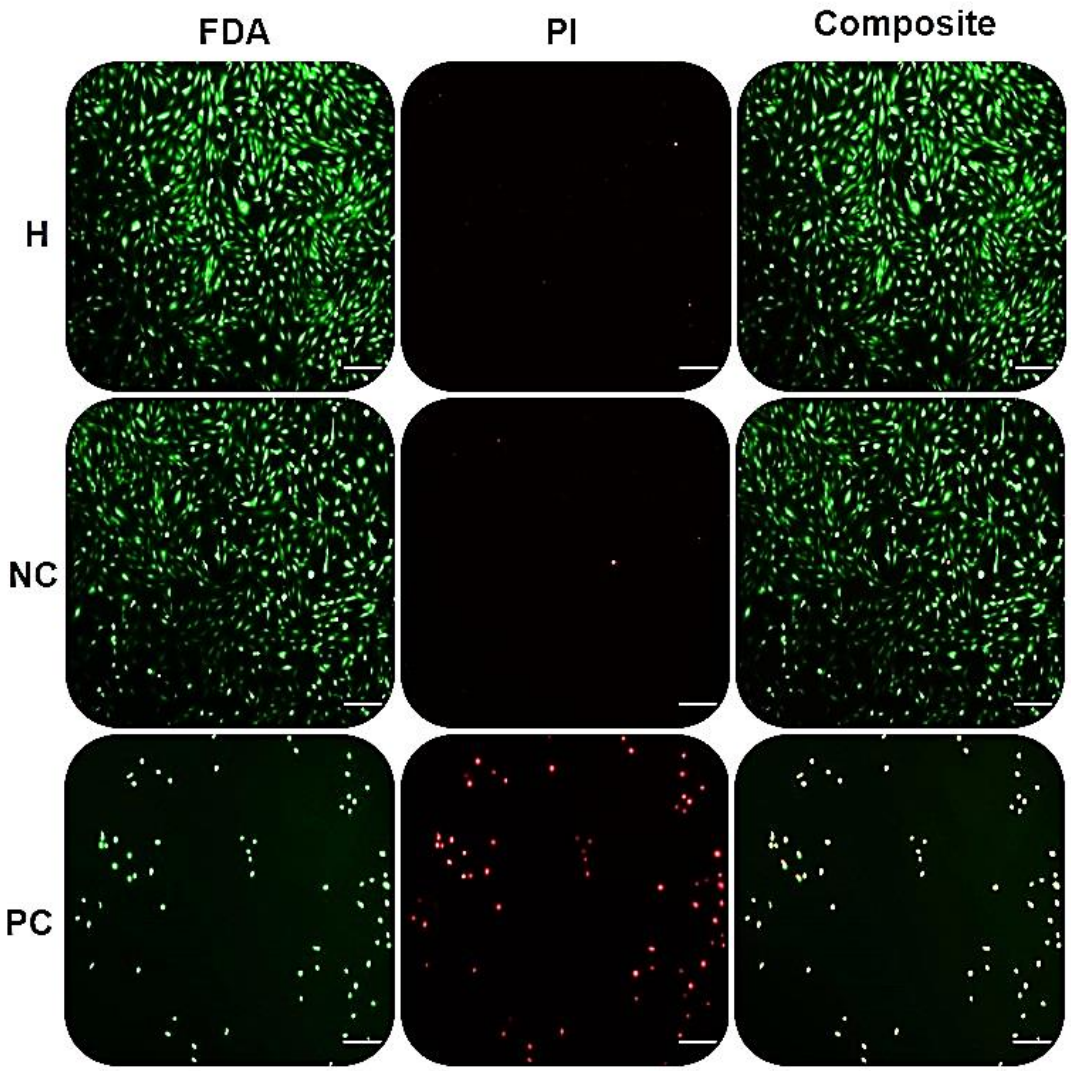

Figure 38: Evaluation of the cytotoxicity of *E. senegalensis* extracts in SC-1 fibroblasts using live-dead staining. H, hexane extract; NC, negative control; PC, positive control; FDA, fluorescein diacetate; PI, propidium iodide. 5x magnification; scale bar: 200 µm

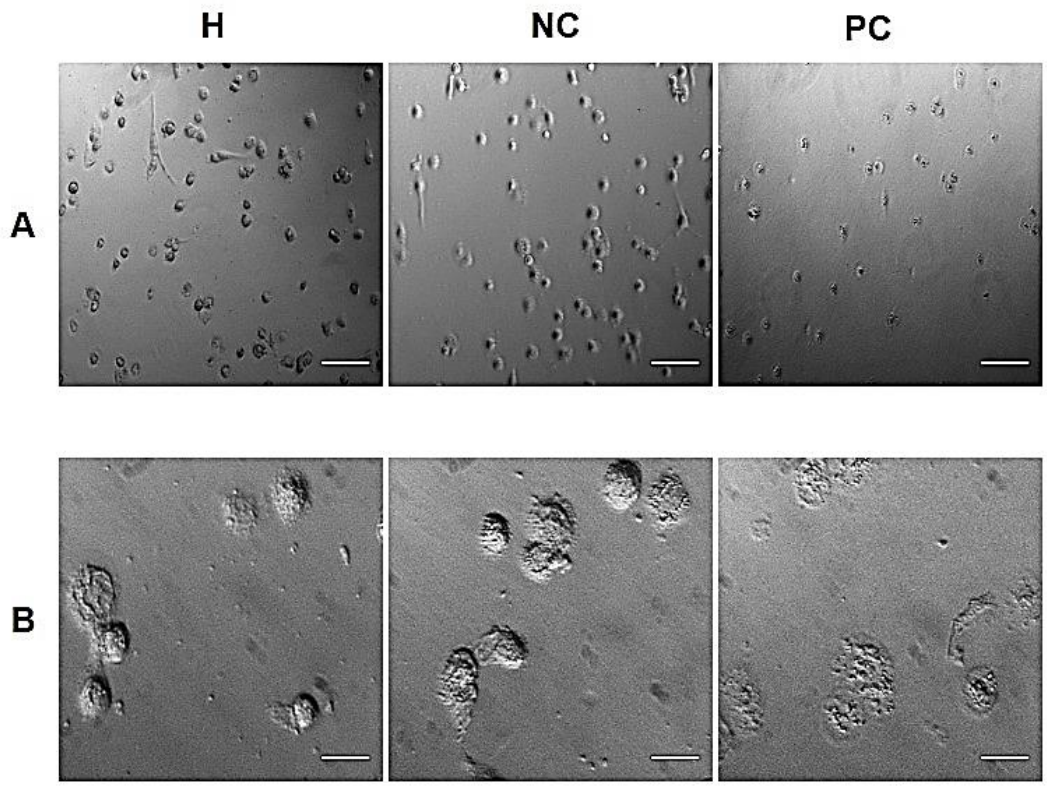

Figure 39: Phase contrast (**A**) and PlasDIC (**B**) images of THP-1 macrophages exposed to 100 µg/mL of *E. senegalensis* extracts. H, hexane extract; NC, negative control; PC, positive control. Images were captured at 10× (**A**) and 40× magnification (**B**); scale bars: 100 µm (**A**) and 25 µm (**B**).

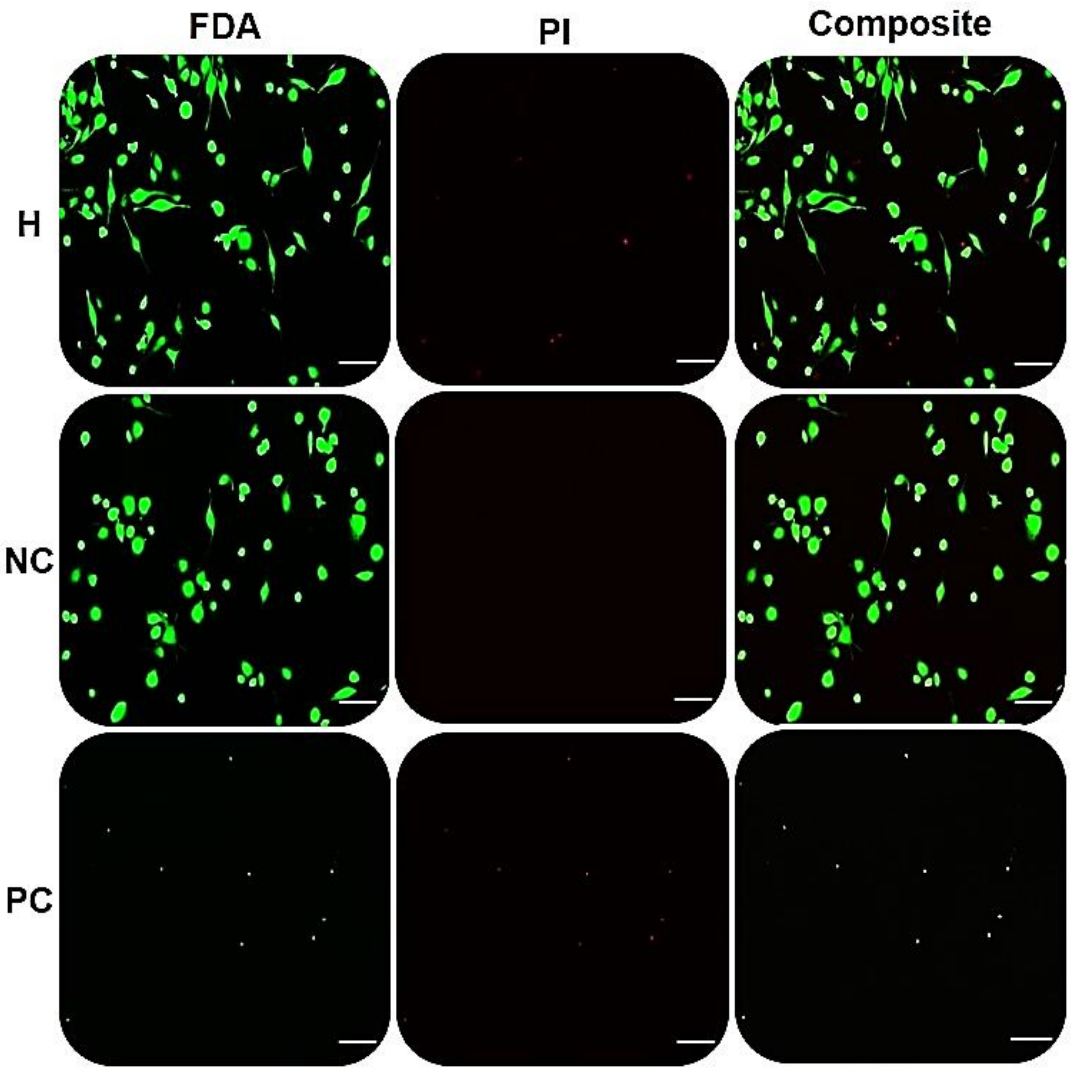

Figure 40: Evaluation of the cytotoxicity of *E. senegalensis* extracts in THP-1 macrophages using live-dead staining. H, hexane extract; NC, negative control; PC, positive control; FDA, fluorescein diacetate; PI, propidium iodide. 10x magnification; scale bar: 100 µm.

Overall, however, the methanol extract of the plant had the greatest cytotoxic effect in the three cell lines tested. This could be ascribed to the presence of both neobavaisoflavone and rotenone (Figure 19). The cytotoxic potential of neobavaisoflavone has already been discussed earlier.[114] On the other hand, rotenone is widely known for its cytotoxic effects through the induction of oxidative stress and apoptosis.[152, 153] Hence, these two compounds could partly explain the decreased effect of the extract on cellular density.

The findings of this study are generally in consonance with previous publications. Although no literature could be obtained regarding cytotoxicity assessment of extracts prepared from *E. senegalensis* in myoblasts, fibroblasts, and monocytes, dichloromethane extracts of the root-bark of the plant has reported moderate toxicity in a brine shrimp toxicity assay, with 73% dead larvae at 50 µg/mL.[154] Aqueous extracts of the stem-bark displayed no cytotoxic effect in mouse embryonic stem cells.[82] Also, a wide margin of safety was recorded in an acute and sub-chronic study of a decoction prepared from the stem-bark.[155] Interestingly, however, flavonoids isolated from plants of the genus *Erythrina* have been found to be selectively cytotoxic in some cancer cells. Kuete *et al*. isolated two isoflavonoids and a pterocarpan from *E. senegalensis* which showed significant cytotoxicity against a host of cancer cells.[114] This suggests the possible presence of some cytotoxic compounds in extracts prepared from *E. senegalensis*. The current study has confirmed the presence of neobavaisoflavone, as well as the possible presence of rotenone in extracts of the plant. Even though the present study and others have shown extracts to have minimal or no cytotoxic potential in normal mammalian cells, the presence of the two compounds could result in cytotoxic manifestations at higher concentrations.[114, 152, 153]

Furthermore, this study has demonstrated that ethnomedicinal preparations of the plant could promote proliferation of THP-1 monocytes. No *Erythrina spp*. has yet been reported to enhance proliferation of these cells. However, other plants from the family Papilionaceae, such as *Butea monosperma,* have been found to increase cell proliferation and collagen synthesis.[156] Therefore, it is possible that the ethnomedicinal extract could possess compounds which enhance cell proliferation, thereby increasing cell density. This could add credence to the traditional use of the plant as a wound healing remedy, as increased proliferation of macrophages will be relayed to increased healing.

3.6. Conclusion

The SRB assay and three different microscopic techniques were used to assess the cytotoxic potential of extracts. Whilst the extracts were generally non-cytotoxic, some displayed marginal cytotoxicity especially at higher concentrations (100 µg/mL). The most cytotoxic extracts to the myoblasts, fibroblasts, and macrophages were the

methanol and ethnomedicinal extracts of *A. africana*, and the hexane extract of *E. senegalensis*, respectively. Comparatively, the macrophages were the least affected by extract exposure, whilst the myoblasts were the most affected. Although the concentrations shown to have marginal cytotoxic effect (100 µg/mL) is not likely to be achieved systemically, it is easily achievable topically. Therefore, extracts from the plant should be used cautiously in wounds.

Chapter 4

Antioxidant and anti-inflammatory activity of extracts

4.1. Introduction

4.1.1. Relevance of reactive oxygen species and antioxidants in wound healing

Radical derivatives of oxygen and other free radicals, such as reactive nitrogen species (RNS), play pivotal roles in the wound healing process.[157] Familiar members of the ROS family, such as the OH•, H_2O_2, and O_2•, contribute in diverse ways throughout the healing process to enhance healing.[158] Wounding results in loss of blood from the injured site. This triggers release and activation of various factors, including ROS and RNS, which act as secondary signalling molecules to facilitate haemostasis by regulating vascular constriction and vasodilation, respectively.[157, 158] By doing so, blood volume is restored and the risk of infection minimized. This enables other wound healing processes such as the inflammation and proliferation of cells to occur unimpeded.[5]

The process of inflammation is aimed at keeping a sterile wound environment for cellular proliferation and other wound healing events to occur.[5, 18] ROS is crucial for the success of the inflammatory phase.[157, 159] Apart from being directly involved in the inflammatory phase of the healing process, ROS participates in wound healing by enhancing activity of phagocytic cells, like macrophages and neutrophils, by increasing influx of potassium into phagocytic vacuoles.[157] As a result, optimal pH is maintained for activity of granule proteases. Secondly, ROS induces the expression of macrophage chemoattractant proteins, such as macrophage inflammatory proteins 1α and 2, for phagocyte recruitment. Also, it causes the elevation of cellular adhesion molecules such as leukocyte function-associated antigen-1 which assists in leukocytoplania. Furthermore, it serves as a conduit for inducing the production of various ROS and other inflammatory proteins.[157, 159]

The role of ROS in cell proliferation and other stages of healing are equally well-documented. The upregulation of a ROS-producing protein, NADPH oxidase-4, leads to the induction of myofibroblast activation, migration and proliferation.[160] This results in enhanced healing of wounds in diabetic mice.[160] Several other reports are available

to attest to the significance of ROS in cellular proliferation and collagen deposition for tissue remodelling. These include ROS-induced proliferation of endothelial cells, macrophages, and keratinocytes.[157, 159] However, although ROS is crucial to the healing process, uncontrolled production of ROS is one of the causes of delayed wound healing.[15, 157]

Intracellular ROS is kept at physiological levels by a system of proteins known as antioxidants. These include catalase, SOD and glutathione *S*-transferases.[27] By so doing, antioxidants promote healing by maintaining a healthy redox system. However, when the endogenous antioxidants are overwhelmed, oxidative stress occurs, causing damage to tissues and delaying the healing process.[27, 157] Exogenous antioxidants such as those of plant origin are therefore useful adjuncts to facilitating the healing of such delayed wounds, and therefore continuous research into the discovery of novel antioxidants is encouraged.

4.1.2. Methods for determining antioxidant activity

Several methods have been described for assessing the antioxidant potential of plant extracts (Table 10). In this study, the DPPH and ABTS assays were adapted for acellular analysis of extracts for antioxidant activity. This was because of their suitability and sensitivity in the detection of antioxidants from plant extracts, as well as the ease of conducting the two assays.[161] A combination of the two assays also ensured that both lipophilic and hydrophilic antioxidants in the samples were detected.[161, 162] Secondly, the ability of extracts to attenuate oxidative stress in cells was determined using dichlorofluorescien diacetate (DCF-DA), a fluorescent dye which is capable of detecting intracellular levels of H_2O_2 and other ROS.

Table 10: Methods for determining antioxidant activity.[161]

Method	Principle	Advantage(s)	Disadvantage(s)
DPPH scavenging activity	• Reduction of the stable DPPH free radical (violet) • Loss of colour measured at 517 nm	• Easy, effective, and rapid • Inexpensive	• It is an acellular assay • Less sensitive for lipophilic antioxidants
Hydrogen peroxide scavenging assay	• Measures disappearance of H_2O_2 in the presence of antioxidants at 230 nm	• Simple and easy to do • Inexpensive	• Interference from secondary metabolites with absorbance at 230 nm
Nitric oxide scavenging activity	• Based on the interaction between NO and O_2 to form nitrite ions which yields a chromaphore with Greiss reagent • Antioxidants scavenge NO, leading to reduced production of nitrite ions	• Quantifiable reaction with nitrite	• High detection limit (1 – 2 µM) • Limited to In vitro analysis • Several sample preparation steps
ABTS scavenging assay	• Discoloration of ABTS by antioxidants • Decrease in colour measured at 734 nm	• Simple • Highly sensitive • Useful for both hydrophilic and hydrophobic antioxidants	• Limited to non-cellular assays
Ferric reducing-antioxidant power	• Reduction of ferric iron to the ferrous form by antioxidants • Measured at 593 nm	• Simple • Inexpensive • Measures antioxidant potential in a wide range of samples	• Low precision

DPPH, 2,2-diphenyl-1-picrylhydrazyl; H_2O_2, hydrogen peroxide; NO, nitric oxide; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid).

4.1.3. Inflammation and wounds

The inflammatory process is pivotal to the successful healing of wounds (as described in Chapter 1, Figure 2). Briefly, this is triggered by the infiltration of the wound vicinity by neutrophils, macrophages, and other inflammatory cells in response to chemotactic proteins, such as prostaglandins, IL-1, TNF-α, and TGF-β.[5] Neutrophils defend the

wound from bacteria and remove tissue debris by releasing several types of proteolytic enzymes and ROS.[157] It also secretes TNF-α which amplifies neutrophil chemotaxis and facilitates the other stages of healing. The role of macrophages is equally important to the inflammatory and other phases such as the proliferation of various cells involved in wounds. Like neutrophils, they are also involved in wound debridement and sterilization through their phagocytic action.[5, 157] Additionally, they serve as source of various growth factors and cytokines required for other processes, including angiogenesis and wound remodelling.[5]

Although inflammation is pivotal to wound healing, its prolongation has been indicated as one of the causes of delayed healing. Fluid derived from wounds with delayed healing has elevated amounts of pro-inflammatory cytokines, such as TNF-α, and IL-1β. However, the levels of these cytokines decrease as the wound starts healing, indicating a close correlation between non-healing wounds and inflammation.[6] Subsequently, anti-inflammatory therapy has been suggested to facilitate healing of chronic wounds. Topical application of infliximab (TNF-α inhibitor), in chronic ulcer patients who had failed to respond to other available conventional treatments, resulted in a significant improvement in healing within four weeks of treatment.[163] Also, the wound healing effect of some plant extracts and phytochemicals has been linked to their anti-inflammatory properties. The ability of curcumin, a wound healing phytocompound, to stimulate healing has been attributed to its potential to decrease inflammatory cytokines, while increasing anti-inflammatory cytokine levels.[164]

Therefore, experiments targeted at discovering alternative anti-inflammatory treatments must be encouraged. In the current study, the plant extracts were assessed for ability to inhibit inflammation, using the XO enzyme as a marker.

4.1.4. Relationship between xanthine oxidase and inflammation

Xanthine oxidase is an enzyme which uses biologically active nucleotides emanating from nucleic acids and nucleotide mediators to catalyse the two terminal reactions of purine catabolism in humans, primates and birds.[165] It has two inter-convertible isoforms, an oxygen-dependent oxidase and a nicotinamide adenine dinucleotide (NAD^+)-dependent dehydrogenase. Both isoforms play central roles in the conversion of hypoxanthine to xanthine, and then further into uric acid (Figure 41).[165] The end

product, uric acid, is largely excreted via the kidneys and intestinal tract. However, the reaction generates ROS, such as the superoxide radical and H_2O_2, which could cause undesirable effects. Consequently, overactivity of XO results in a condition known as gout, a common rheumatic disease and an acute inflammatory arthritis, characterised by high circulating uric acid levels.[166] The inhibition of XO therefore has the potential to reduce both oxidative stress and circulating levels of uric acid.

Recent evidence suggests a major role of XO in the inflammatory process. Subsequent to the expose that XO activity increases in diabetic animals, it was found that the activity of the enzyme results in an increase in various inflammatory proteins and cytokines, such as NF-κB, IL-1β, iNOS, and IL-6.[167] Moreover, these events were abolished by the XO inhibitor, allopurinol.[167] In a related study, allopurinol protected rodents from renal ischemia by downregulating TNF-α, IL-1β, and IL-6, decreasing neutrophil activity and enhancing antioxidant capacity.[168] Furthermore, the pro-inflammatory and growth factor stimulation of human myeloid cells has been reported to cause XO activation.[165]

Considering the role played by both ROS and inflammation in the pathophysiology of wounds, and their relationship with XO activity, inhibitors of this enzyme could be useful in the wound healing process. Moreover, elevated levels of uric acid have been found in wound fluid of chronic venous leg ulcers, with a concomitant depletion in uric acid precursors such as adenosine in impaired wounds.[169] Also, an increased activity of XO was observed in chronic wound fluids, suggesting a correlation between the enzyme activity and wound severerity.[169] As a result of these revelations, there have been major research activities into identification of inhibitors of the enzyme for therapeutic reasons.

Some crude plant extracts and phytoconstituents like flavonoids and polyphenolic compounds have been reported to possess XO inhibitory activity. The leaf extract of *Amaranthus viridis*, a plant traditionally used for managing ulcers, inhibited XO activity.[170] This effect was attributed to its anti-β-Hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase, antioxidant, and anti-inflammatory activities.[170] Three phenolic compounds, chlorogenic acid, hyperin, and quercetin, improved endothelial function by inhibiting XO, and oxidative damage.[171] The compounds also decreased

pro-inflammatory cytokines induced by TNF-α, including intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and monocyte chemotactic protein-1.[171] These examples indicate the potential that medicinal plants could have on XO, and hence their therapeutic potential on medical conditions such as wounds, where the enzyme has been implicated.[166]

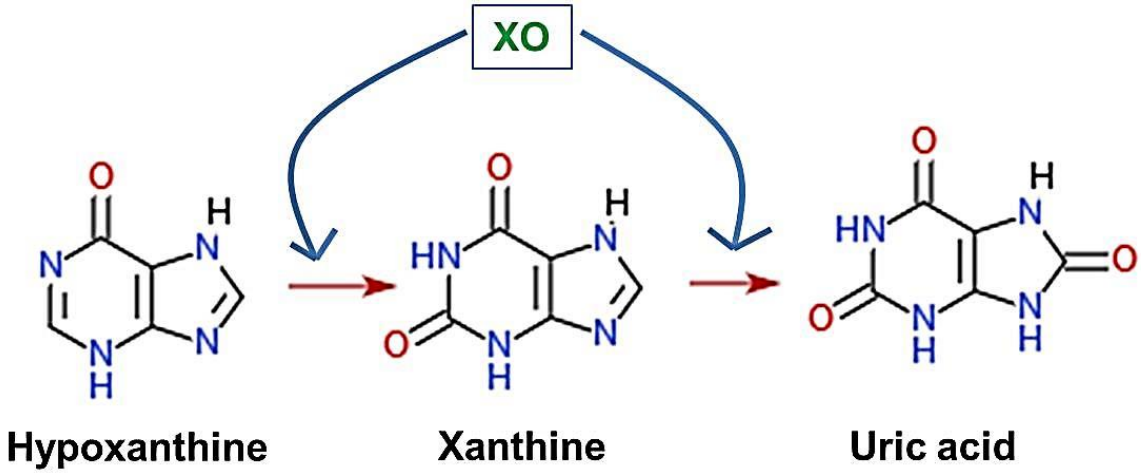

Figure 41: Xanthine oxidase-induced uric acid synthesis. XO: xanthine oxidase.

4.2. Aim and objectives of chapter

This chapter was aimed at assessing the plant extracts for possible antioxidant and anti-inflammatory effects.

The objectives were to:

- determine the antioxidant potential of extracts by exploring their ability to scavenge the ABTS and DPPH free radicals.
- evaluate the extracts ability to suppress oxidative stress in SC-1 fibroblasts, C2C12 myoblasts, and THP-1 differentiated macrophages.
- examine the potential anti-inflammatory effect of extracts using XO activity as a marker.

4.3. Methodology

4.3.1. Antioxidant activity determination

4.3.1.1. ABTS radical scavenging activity

The ABTS radical scavenging effect of extracts was estimated using the method of Re *et al*.[162] 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), and 0.6 – 5.0 µg/mL Trolox (in-reaction, antioxidant control) in a 96-well plate. Equivalent volumes of distilled water (ethnomedicinal and water extracts) and 1% DMSO in methanol (organic extracts, in-reaction) served as negative control. 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:

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

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

4.3.1.2. DPPH radical scavenging activity

The DPPH radical scavenging effect of extracts was estimated using the method of Manzocco *et al*.[172] 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 prior to experimentation. An aliquot of 180 µL DPPH solution was mixed with 20 µL of varying concentrations of the extracts (20 – 320 µg/mL in-reaction), and 0.6 – 5.0 µg/mL Trolox (in-reaction, antioxidant control) in a 96-well plate. Equivalent volumes of distilled water (ethnomedicinal and water extracts) and 1% DMSO in methanol (organic extracts, in-reaction) served as negative control. 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 extracts to scavenge the DPPH radical was calculated using the equation above (Section 4.3.1.1).

4.3.1.3. Determination of effect on oxidative stress

The ability of extracts to alter intracellular ROS was assessed in SC-1 fibroblasts, C2C12 myoblasts, and THP-1 differentiated macrophages, using the AAPH-induced oxidative stress model as described by Lopachev *et al*.,[173] 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 at 37ºC and 5% CO_2. 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 H_2-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 and vehicle controls to which equivalent volumes of 5 µg/mL Trolox and 1% DMSO (PBS for water and ethnomedicinal extracts) were added, respectively. Cells were washed twice with 100 µL PBS and all wells exposed to 50 µL PBS containing 100 µM AAPH, except for the negative controls to which 50 µL PBS alone 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:

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

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

4.3.2. Evaluation of xanthine oxidase activity

The enzyme XO catalyzes the oxidation of xanthine to uric acid. During this reaction, molecular oxygen acts as an electron acceptor, producing superoxide radicals according to the following equation:[174]

$$\mathrm{Xanthine} + \mathrm{O_2} + \mathrm{H_2O} \rightarrow \mathrm{Uric\ acid} + \mathrm{O_2} + \mathrm{H_2O_2}$$

Xanthine oxidase activity was evaluated under aerobic conditions[174] by the spectrophotometric measurement of the production of uric acid from xanthine. Extracts were assayed for their XO inhibitory effect using previously reported methods with some modification.[175] The assay mixture consisting of 500 µL of the plant extracts at various concentrations (3 – 100 µg/mL in phosphate buffer, in-reaction), 350 µL of 0.1 M phosphate buffer (pH = 7.5), and 300 µL enzyme solution (0.02 units/mL in phosphate buffer, in-reaction) was prepared immediately before use. Allopurinol (1.5 µg/mL – 100 µg/mL, in-reaction) served as positive control, whilst phosphate buffer served as the negative control. Equivalent volumes of 1% DMSO (in-reaction) and buffer were used as vehicle controls for the organic and aqueous extracts, respectively. After pre-incubation at 25^{o}C for 15 min, the reaction was initiated by the addition of 600 µL of substrate solution (300 µM xanthine in phosphate buffer, in-reaction). The assay mixture was incubated at 25^{o}C for 30 min, and the reaction stopped by adding 250 µL of 1 M HCl. The inhibition of XO activity was followed by measuring the increase of uric acid absorbance at 290 nm as proposed by Cimanga *et al*.[176] Appropriate colour controls were included by adding extracts (500 µL) to phosphate buffer (1500 µL) and measuring absorbance at 290 nm. Xanthine oxidase inhibitory activity was determined using the equation:

$$Xanthine\ oxidase\ activity\ (\%\ relative\ to\ negative\ control) = \frac{Ac - As}{As} \times 100$$

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

4.4. Statistical analysis

Data represents results of at least three independent experiments conducted with technical triplicates. Statistical analysis was performed using GraphPad Prism 7 data analysis software. Data was expressed as the mean ± SEM. Non-linear regression was used to determine half-maximal inhibitory concentrations (IC_{50}). The difference between groups was determined by a Kruskal-Wallis test followed by Dunn's post-hoc test. *P* values less than 0.05 were considered significant.

4.5. Results and discussion

4.5.1. Antioxidant activity

The DPPH and ABTS assays have been widely used to determine the antioxidant activity of various plants and pure compounds. Both DPPH and ABTS form stable free radical solutions which show characteristic absorptions at 515 nm and 734 nm, respectively.[162, 177] These free radicals are reduced in the presence of hydrogen donating compounds such as antioxidants, thereby causing a reduction in colour intensity of the free radical solution.[162, 177] In the current study, Trolox, a known antioxidant compound, was used as the positive control to quench the ABTS free radical, yielding an IC_{50} of 2.92 µg/mL (Figure 42 and Table 11). This was comparable to the IC_{50} of Trolox in literature (2.5 µg/mL),[178] an indication that the assay worked optimally.

Most of the extracts exhibited good activity against ABTS, with IC_{50} values below 100 µg/mL (Figure 42 and Table 11). However, the most active extract against the free radical was the ethyl acetate extract of *B. diffusa*. The IC_{50} recorded for the extract was 21.23 µg/mL. This was followed by the ethnomedicinal extract of *A. africana*, with an IC_{50} of 36.18%. The ethyl acetate extract of *A. africana*, and the water and ethnomedicinal extracts of *B. diffusa* exhibited moderate activity against the free radical (IC_{50} > 100 µg/mL). Comparatively, the hexane extracts of all three plants exhibited the weakest activity against ABTS. The maximum scavenging effect upon treatment with the hexane extracts (21.6%) was obtained from the *B. diffusa* hexane extract. Comparing the three plants, extracts from *E. senegalensis* had the most prominent effect against the free radical, with all extracts (except hexane) having IC_{50}s below 100 µg/mL.

Whereas the water and ethnomedicinal extracts of *B. diffusa* and *E. senegalensis* recorded comparable ABTS radical scavenging effects, the ethnomedicinal extract of *A. africana* was more efficacious that its water extract. Since both extracts were obtained from water, the results suggest that the partial fractionation of compounds as a result of the sequential extraction has resulted in the decreased antioxidant activity of the water extract. It also indicates that the ABTS radical scavenging effect of the ethnomedicinal extract of the plant results from an additive or synergistic effect of the antioxidant compounds present.

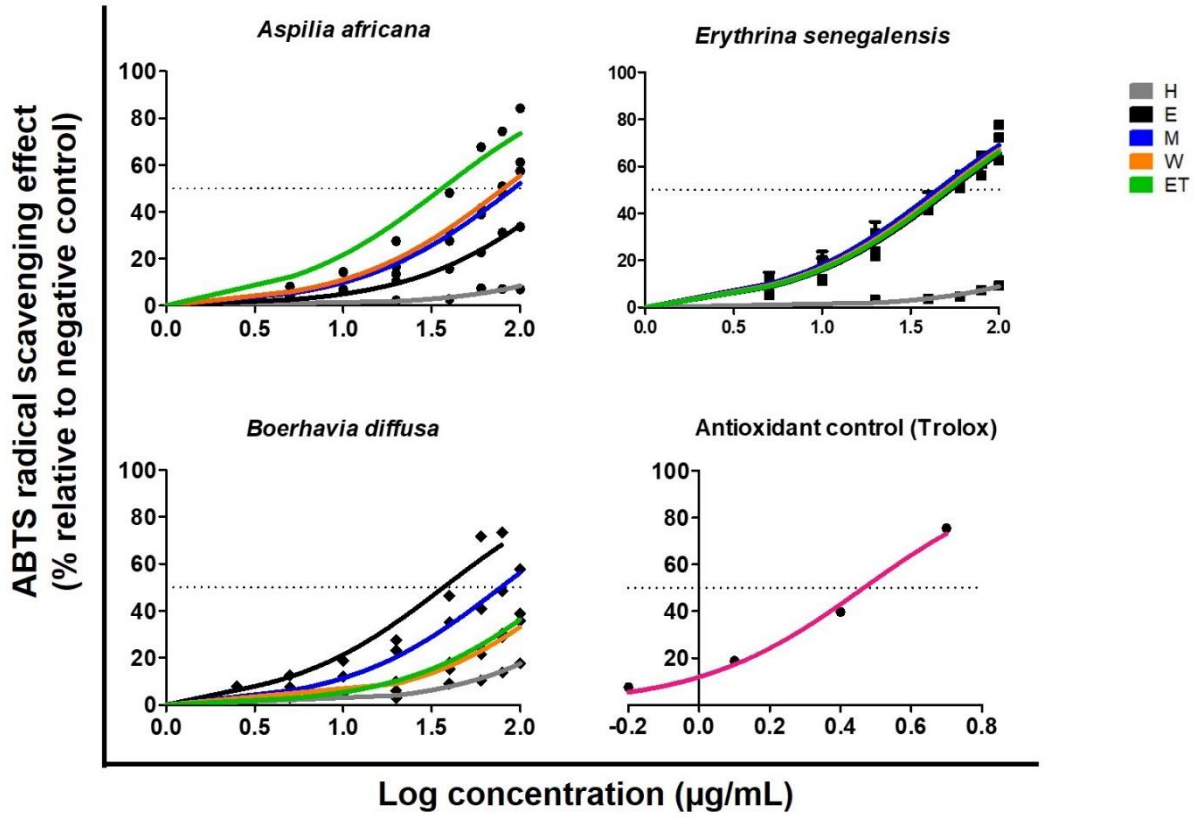

Figure 42: ABTS scavenging effect of extracts from *A. africana*, *B. diffusa*, and *E. senegalensis*. H: hexane; E: ethyl acetate; M: methanol; W: water; ET: ethnomedicinal extracts

In the DPPH radical scavenging assay, Trolox caused the desired discolouration of the free radical solution, yielding an IC_{50} of 6.27 µg/mL (Table 11). This was comparable to the IC_{50} of Trolox against DPPH in published data (6.1 µg/mL).[178] Compared to the ABTS radical scavenging ability, the extracts had lower activity against DPPH (Figure 43). As a result, the concentration of the extracts was increased to a maximum of 320 µg/mL.

The most active extracts were the methanol extracts of *A. africana* (IC_{50} = 278.00 µg/mL) and *E senegalensis* (IC_{50} = 291.1 µg/mL). Though the ethyl acetate, water, and ethnomedicinal extracts of the two plants showed signs of activity at higher concentrations, all the *B. diffusa* extracts poorly scavenged DPPH. Even the extract with the greatest ABTS radical scavenging effect, the ethyl acetate extract of *B.*

diffusa, recorded weak DPPH scavenging effect (Table 11). Furthermore, none of the hexane extracts had activity against the free radical.

Table 11: Antioxidant activity of extracts from *Aspilia africana*, *Boerhavia diffusa*, and *Erythrina senegalensis.*

Medicinal plant	Extract	IC_{50} (µg/mL)	
		ABTS	DPPH
Aspilia africana	Hexane	> 100	> 320
	Ethyl acetate	> 100	> 320
	Methanol	91.66 ± 1.03	278.00 ± 2.44
	Water	80.51 ± 1.03	> 320
	Ethnomedicinal	36.18 ± 1.05	> 320
Boerhavia diffusa	Hexane	> 100	> 320
	Ethyl acetate	21.23 ± 1.03	> 320
	Methanol	77.72 ± 1.02	> 320
	Water	> 100	> 320
	Ethnomedicinal	> 100	> 320
Erythrina senegalensis	Hexane	> 100	> 320
	Ethyl acetate	53.29 ± 1.07	> 320
	Methanol	44.86 ± 1.03	291.10 ± 2.46
	Water	49.05 ± 1.04	> 320
	Ethnomedicinal	51.35 ± 1.03	> 320
Trolox		2.92 ± 1.02	6.27 ± 1.07

The findings indicate that the antioxidant components within the extracts do not scavenge the two free radicals equally. The extract with the most pronounced ABTS radical scavenging activity (ethyl acetate extract of *B. diffusa*) yielded weak DPPH radical scavenging activity. On the other hand, the methanol extracts of *A. africana* and *E. senegalensis,* which recorded moderate ABTS radical scavenging effects, had better activity against the DPPH radical. Several factors may account for the varied scavenging ability of the extracts against the two free radicals. Some antioxidant components may selectively quench one free radical, and not the other. This is because the radicals have different stereoselectivity, resulting in different electron and hydrogen atom transfer potentials.[179] The solubility of extracts in the two free radical solutions may also differ, and thereby contribute to the variability. Furthermore, the DPPH free radical is known to be scavenged poorly by lipophilic antioxidants.[161] This

could explain why antioxidants in the ethyl acetate extract of *B. diffusa* poorly scavenged the free radical even though it strongly scavenged the ABTS radical.

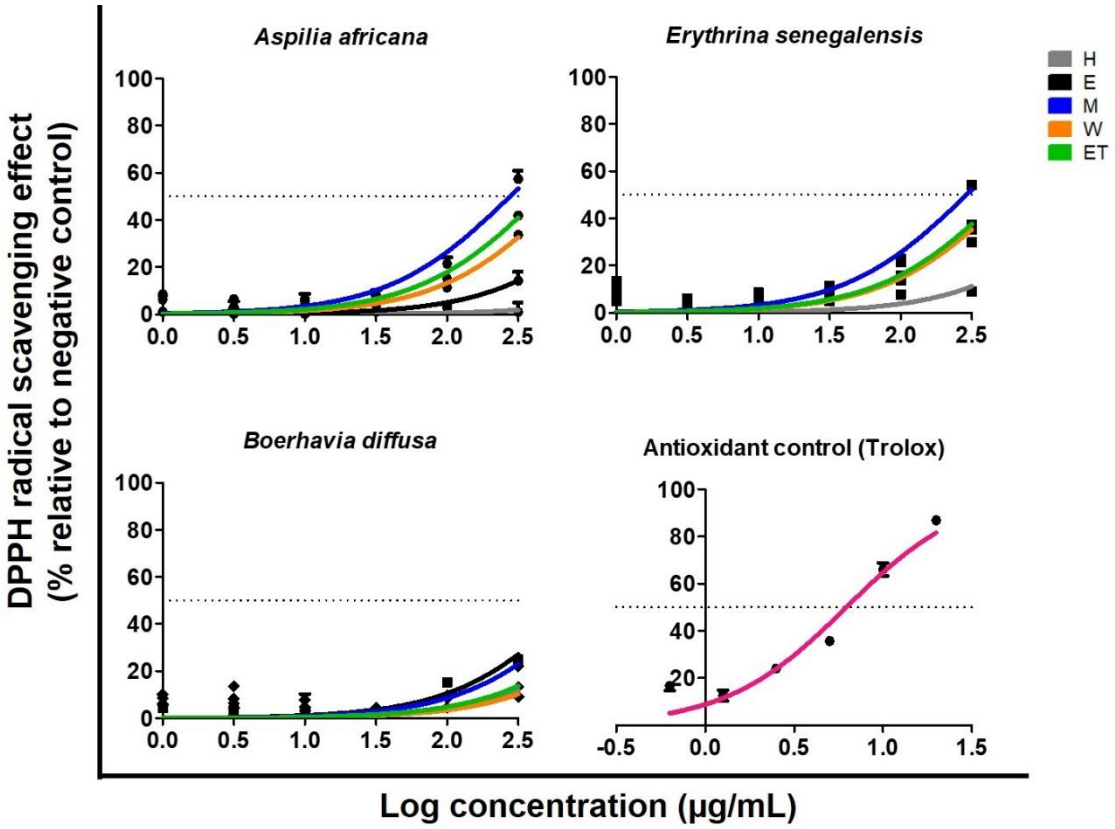

Figure 43: DPPH scavenging effect of extracts of *A. africana*, *B. diffusa*, and *E. senegalensis*. H: hexane; E: ethyl acetate; M: methanol; W: water; ET: Ethnomedicinal extracts

No published data on ABTS scavenging effect of extracts prepared from either *A. africana* or *E. senegalensis* could be found in literature. Some previous studies on *B. diffusa* were, however, noted. The aqueous, ethanolic and hydroethanolic extracts of the roots of *B. diffusa* recorded IC_{50}'s of 53.4 µg/mL, 44.84 µg/mL and 43.62 µg/mL, respectively against ABTS.[180] No ethanolic extracts were used in the current study. Even though methanol is similar to ethanol, the methanol extract used in the current study was obtained by sequential extraction. Also, both the water and ethnomedicinal extracts of the plant yielded minimal ABTS radical scavenging effects (IC_{50} > 100 µg/mL). The difference in findings between the two studies could be because of the different plant parts, sampling sites, and storage conditions used. Whilst the current study used extracts prepared from whole plants of *B. diffusa*, the previous study

sampled the roots. This indicates that the roots of *B. diffusa* may have a higher antioxidant component than the aerial parts. A study on *B. procumbens*, a related species, found high ABTS scavenging activity of the ethyl acetate extract (IC_{50} = 46.7 µg/mL).[181] However, minimal activity was recorded for the methanol, n-hexane, n-butanol and aqueous extracts of the plant (IC_{50} > 100 µg/mL).[181] Although the hexane and aqueous extracts had low activity in the current study, both the ethyl acetate and methanol extracts had better scavenging effects. These extracts recorded IC_{50} values of 21.2 and 77.7 µg/mL, respectively. Whilst the difference in findings may be due to the different plant species investigated, it could also be a result of the differences in extraction technique. The previous study involved the use of crude extracts,[181] whilst extraction was sequential in the current.

Limited data exists regarding the DPPH radical scavenging effect of *A. africana*. The methanol leaf extract of the plant's flower was reported to have 96.8% DPPH radical scavenging activity at 250 µg/mL, when compared with ascorbic acid.[182] This differs considerably from the current findings, where an IC_{50} of 278 µg/mL was recorded for the methanol extract. This could be due the use of crude extraction in the previous study, as well as the different parts used.[182] Recently, an antioxidant compound isolated from the n-butanol fraction of the methanol extract of the leaf (parahydroxybenzaldehyde) was reported.[183] This compound had better DPPH radical scavenging activity than both the n-butanol fraction and methanol extracts. The findings of the present study indicate that the methanol extract of the plant has better ability to quench DPPH radicals, compared to the other extracts. This is generally in agreement with the two previous findings.

Unlike *A. africana*, there are several previous accounts of DPPH radical scavenging effects of extracts from *B. diffusa*. The n-hexane, ethyl acetate, and methanol extracts of the plant obtained by continuous Soxhlet extraction demonstrated high radical scavenging effects.[184] The methanol extract showed higher activity (IC_{50} = 8.18 µg/mL), than the n-hexane (IC_{50} = 40.61 µg/mL) and ethyl acetate extracts (IC_{50} = 43.81 µg/mL). This is contrary to the current findings. Results from the present study indicate poor DPPH scavenging activity of all five extracts from the plant. This variation in findings could be due to the different extraction techniques used. In a related study, the methanol root extract was reported to have better DPPH radical scavenging

activity (IC50 ≈ 50 µg/mL), compared to the ethanol and aqueous extracts (IC_{50} ≥ 250 µg/mL).[185] These findings attest to the weak DPPH radical scavenging effect of extracts prepared from the plant, as was found in the current study.

The dichloromethane extract of *E. senegalensis* root and stem-bark were found to yield poor DPPH radical scavenging activity.[154] A later study, however, reported significant activity with the dichloromethane and ethyl acetate extracts of these parts of the plant (IC_{50} < 10.0 µg/mL).[186] The initial defatting of powdered plant material by ether before extraction could account for the reason why the findings of the latter study differed from the earlier account.[154, 186] Even though the sequential extracts used in the current study were potentially defatted by the prior use of hexane, the ethyl acetate extract recorded poor DPPH radical scavenging effect (IC_{50} > 320 µg/mL). The difference in findings between the current and previous studies could be ascribed to the different plant parts used.

Multiple studies have been carried out to determine the antioxidant activity of extracts from *B. diffusa* and *E. senegalensis,* albeit using other antioxidant methods. A superior antioxidant activity of the ethyl acetate and aqueous extracts of *B. diffusa* leaves (IC_{50} < 10 µg/mL) against H_2O_2 and hypochlorous acid compared to N-acetylcysteine was reported.[187] Boeravinone G, a rotenone isolated from the roots of *B. diffusa*, has been shown to inhibit thiobarbituric acid reactive substances (TBARS) from Caco-2 cells, and ROS formation induced by Fenton's reagent.[188] The compound also increased SOD, and reduced H_2O_2-induced DNA damage.[188] In a study to determine the hepatoprotective and antioxidant activity of the hydroethanolic extract of *E. senegalensis* stem-bark, a positive correlation was reported between the two bioactivities.[189] The extracts were assessed for antioxidant potential using the DPPH radical scavenging activities, ß-carotene-linoleic acid model system (ß-CLAMS), ferric-reducing antioxidant power (FRAP) assay and microsomal lipid peroxidation (MLP), with activity being comparable to the standard antioxidant , vitamin C.[189] The findings of the current study, therefore, support the rich antioxidant potential of the plants, particularly *B. diffusa* and *E. senegalensis*.

Phytochemical analysis of extracts prepared from the three plants indicated the presence of diverse phytochemical groups, including alkaloids, flavonoids and

phenols, which are known to have antioxidant activity. Specifically, UPLC-MS evaluation of extracts led to identification of some phytochemical compounds or their derivatives in all the extracts. The water and ethnomedicinal extracts of *A. africana* were found to possess ascorbic acid and quercetin, respectively (Figure 14). Both compounds are known to have antioxidant activities, and therefore could be responsible for the observed antioxidant effects of the respective extracts.[190, 191] Though the methanol extract contained several compounds, as evidenced by the numerous peaks on the chromatogram, none of them could be identified in this study. Hence the potential source of the antioxidant activity observed with the extract is yet to be determined.

Three antioxidant compounds, rutin,[191] kaempferol[192] and quercetin,[191] were detected in the *B. diffusa* extracts. While the observed antioxidant effect of the ethyl acetate extract could be due to rutin, the effect in the methanol extract of the plant could be a result of the presence of rutin, kaempferol and quercetin. Similarly, two of these antioxidants were detected in the *E. senegalensis* extracts, rutin and kaempferol (Figure 19). Whereas rutin or its derivatives may be responsible for the observed antioxidant effect in the ethyl acetate and methanol extracts of this plant, kaempferol could also contribute to the effect in the methanol treatment. Though the water and ethnomedicinal extracts of *E. senegalensis* also demonstrated appreciable ABTS scavenging activity, none of the antioxidant marker compounds were detected in either extract.

4.5.2. Effect on AAPH-induced oxidative stress

Oxidative stress, characterised by elevated ROS, is regarded as one of the factors that delays wound healing.[157] In this study, extracts were evaluated for their ability to suppress oxidative stress in three different wound-related cell lines, fibroblasts, macrophages, and myoblasts. Oxidative stress was induced with AAPH, a water-soluble compound with the ability to generate ROS.[173] Exposure to 100 µM AAPH for 2 h led to a fold increase of 2.82, 1.70, and 1.57 in intracellular ROS of the myoblasts, fibroblasts, and macrophages, respectively (Figure 44). Treatment with the antioxidant control (Trolox) prior to exposure to AAPH caused a 2.10-, 1.70-, and 1.89-fold decrease in intracellular ROS in myoblasts, fibroblasts, and macrophages, respectively, compared to the AAPH controls. Phagocytic cells such as neutrophils

and macrophages are known to be major sources of ROS through the activities of NADPH oxidases, mitochondrial electron transport chain, and xanthine oxidase.[158] Whilst non-phagocytic cells such as fibroblasts and myoblasts are also capable of generating ROS, they only contribute about a third of the total amounts produced.[158] This could explain why the differentiated THP-1 macrophages were more resistant to the oxidative stress-inducing chemical.

4.5.2.1. *Aspilia africana*

Pre-treatment of C2C12 myoblasts with extracts from *A. africana* before AAPH exposure caused varying effects on intracellular ROS levels. The most prominent effects were observed upon treatment with the methanol and ethnomedicinal extracts of the plant (Figure 44, Table 12). The methanol extract at the lowest tested concentration (1 µg/mL) caused a 1.74-fold decrease in intracellular ROS, compared to the AAPH-control cells. A concentration-dependent decrease in intracellular ROS was observed with the extract treatment, resulting in a 2.33-fold decrease at 100 µg/mL ($p \leq 0.05$), compared to the AAPH-controls. The effect observed upon pre-treatment with the ethnomedicinal extract of the plant was, however, less pronounced. Intracellular ROS of cells pre-treated with this extract had a fold-decrease of 1.66 - 1.77, compared to the AAPH-controls at a concentration range of 1 – 100 µg/mL. Although the other three extracts of the plant produced no significant ($p \geq 0.05$) effects on intracellular ROS, a concentration-dependent decrease in ROS was observed in the hexane extract pre-treated cells, suggesting a possible ability of the extract to inhibit oxidative stress at concentrations above 100 µg/mL. Although such high concentrations are not likely to be achieved systemically, they could be achieved in topical preparations.

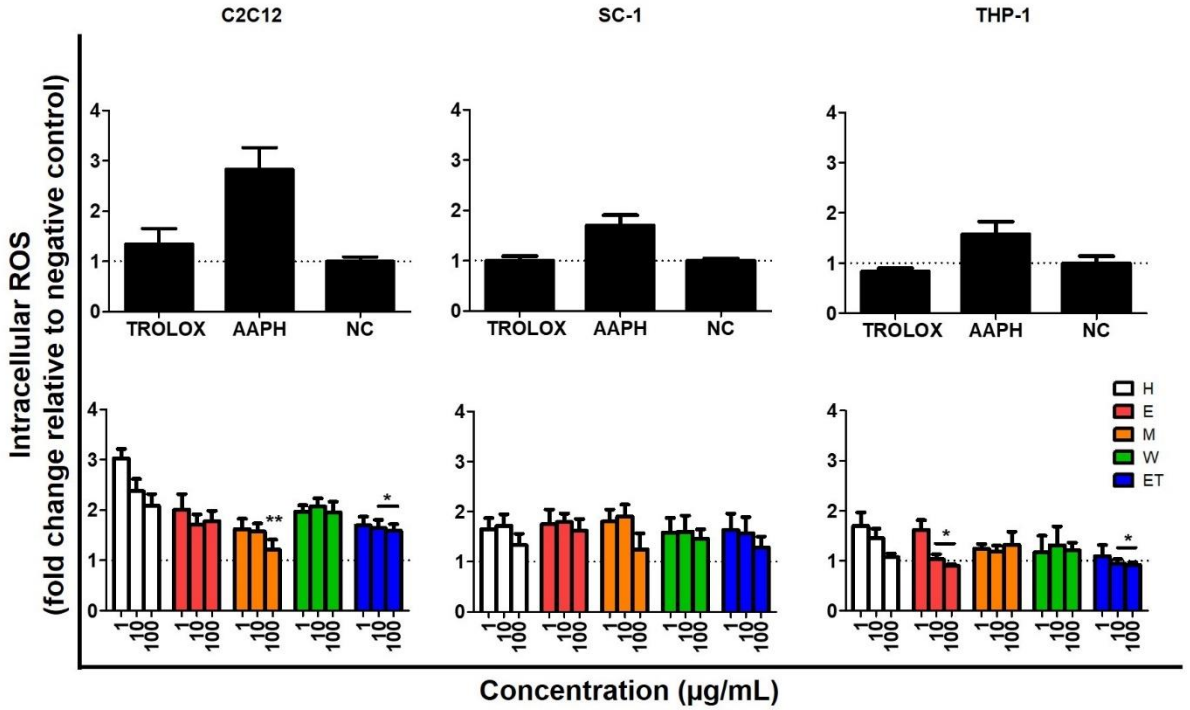

Figure 44: The fold-change in ROS of C2C12 myoblast, SC-1 fibroblast, and THP-1 macrophage cells treated with AAPH after pre-treatment with hexane (H), ethyl acetate (E), methanol (M), water (W) and ethnomedicinal extracts (ET) of *A. africana.* AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride; NC, negative control. Statistical significance relative to AAPH control: $p < 0.05$ (*), $p < 0.01$ (**).

Exposure of SC-1 fibroblasts to extracts of *A. africana* before treatment with AAPH reduced oxidation by various degrees. The most significant effect was observed with the methanol extract. This extract, at 100 µg/mL, caused a fold decrease of 1.37 in intracellular ROS, compared to the AAPH-controls. The other extracts of the plant also resulted in a concentration-dependent decrease in intracellular ROS, albeit less prominent. These four extracts (hexane, ethyl acetate, water, ethnomedicinal), at the highest tested concentration (100 µg/mL), resulted in a 1.28-, 1.06-, 1.16-, and 1.32-fold decrease in intracellular ROS, respectively, compared to the AAPH- controls. Although the effect of the extracts on ROS was insignificant up to 100 µg/mL, the concentration-dependent reduction observed is indicative of a possible role in the protection of fibroblasts against oxidative stress at much higher concentrations. This could be useful for the wound healing process.

The greatest ability of the extracts prepared from *A. africana* to protect against oxidative stress was exhibited in the THP-1 differentiated macrophages. Three of the extracts, the hexane, ethyl acetate, and ethnomedicinal, decreased intracellular ROS generation in a concentration-dependent manner. The three extracts, at 100 µg/mL, caused a 1.47-, 1.74-, and 1.71-fold decrease in intracellular ROS, respectively, compared to the AAPH-controls. This is an indication that the three extracts could protect against oxidative stress, especially at higher concentrations. Interestingly, the effect exhibited by the ethyl acetate and ethnomedicinal extracts were comparable to that of the antioxidant control (Trolox), which caused a 1.89-fold decrease in intracellular ROS at 5 µg/mL. The methanol and water extracts of the plant, however, caused a slight increase in intracellular ROS at concentrations above 10 µg/mL. Intracellular ROS in the two extracts was 1.07- and 1.03-fold higher in cells treated with the 100 µg/mL, compared to the 1 µg/mL. Whilst the extract-induced increase in intracellular ROS could be detrimental to the healing of oxidative stress-related wounds, it could be an adjunct for infection control in other types of wounds.[157]

A close correlation exists between antioxidant activity and suppression of ROS levels.[27, 157] Various phytochemical groups of compounds, such as alkaloids, flavonoids, and phenols, were detected in the plant extracts (Table 6). Specifically, ascorbic acid was detected in the hexane, water, and ethnomedicinal extracts, whilst quercetin was only present in the ethnomedicinal extract (Figure 15). These compounds have confirmed antioxidant activities,[78, 190] hence could account for the observed effect on ROS. Although none of the marker compounds was detected in the methanol extract, its effect implies the presence of some antioxidants or other compounds with effect on ROS. Moreover, the extract exhibited good radical scavenging activity (Table 11; Figures 42 and 43). A search through the literature for the effect of extracts prepared from the plant on intracellular ROS levels revealed that this is the first of such studies for *A. africana*. Further experimentation is therefore required to explore the full impact of the plant on intracellular ROS.

Table 12: Protective effects of extracts against oxidative stress in C2C12 myoblasts, SC-1 fibroblasts, and THP-1 macrophages

Plant name	Extract	Concentration (µg/mL)	Intracellular ROS (fold change) relative to negative control ± SEM		
			C2C12	SC-1	THP-1
Aspilia africana	Hexane	1	3.03 ± 0.19	1.65 ± 0.22	1.70 ± 0.27
		10	2.38 ± 0.24	1.72 ± 0.23	1.45 ± 0.19
		100	2.08 ± 0.24	1.33 ± 0.23	1.07 ± 0.08
	Ethyl acetate	1	2.01 ± 0.31	1.75 ± 0.29	1.62 ± 0.20
		10	1.71 ± 0.20	1.79 ± 0.17	1.03 ± 0.10
		100	1.78 ± 0.21	1.62 ± 0.24	0.90 ± 0.03
	Methanol	1	1.62 ± 0.21	1.80 ± 0.24	1.24 ± 0.10
		10	1.58 ± 0.16	1.90 ± 0.23	1.18 ± 0.12
		100	1.21 ± 0.20	1.24 ± 0.32	1.32 ± 0.27
	Water	1	1.97 ± 0.13	1.58 ± 0.30	1.17 ± 0.34
		10	2.07 ± 0.16	1.60 ± 0.32	1.31 ± 0.38
		100	1.96 ± 0.21	1.47 ± 0.18	1.21 ± 0.15
	Ethnomedicinal	1	1.69 ± 0.18	1.63 ± 0.33	1.09 ± 0.22
		10	1.64 ± 0.16	1.57 ± 0.32	0.94 ± 0.09
		100	1.59 ± 0.13	1.29 ± 0.21	0.92 ± 0.05
Boerhavia diffusa	Hexane	1	3.13 ± 0.48	1.70 ± 0.16	1.68 ± 0.34
		10	1.84 ± 0.31	1.80 ± 0.28	1.24 ± 0.31
		100	1.79 ± 0.27	2.12 ± 0.29	1.20 ± 0.15
	Ethyl acetate	1	2.66 ± 0.51	1.64 ± 0.21	1.49 ± 0.37
		10	1.64 ± 0.34	1.73 ± 0.22	1.05 ± 0.19
		100	1.17 ± 0.24	1.65 ± 0.21	1.08 ± 0.15
	Methanol	1	2.43 ± 0.45	1.66 ± 0.20	1.43 ± 0.17
		10	1.91 ± 0.50	1.90 ± 0.21	1.24 ± 0.14
		100	1.81 ± 0.53	1.63 ± 0.23	1.04 ± 0.12
	Water	1	2.03 ± 0.53	1.72 ± 0.18	1.47 ± 0.22
		10	2.06 ± 0.24	2.10 ± 0.21	1.26 ± 0.34
		100	2.10 ± 0.33	1.91 ± 0.24	1.19 ± 0.19
	Ethnomedicinal	1	1.56 ± 0.23	1.62 ± 0.21	1.16 ± 0.07
		10	1.29 ± 0.21	1.80 ± 0.15	1.25 ± 0.31
		100	1.21 ± 0.17	1.87 ± 0.21	1.03 ± 0.11
Erythrina senegalensis	Hexane	1	2.35 ± 0.41	2.12 ± 0.32	1.28 ± 0.13
		10	1.91 ± 0.33	2.13 ± 0.35	1.28 ± 0.07
		100	1.79 ± 0.28	1.25 ± 0.10	1.17 ± 0.09
	Ethyl acetate	1	1.68 ± 0.36	1.66 ± 0.28	1.13 ± 0.10
		10	1.39 ± 0.32	1.49 ± 0.42	1.17 ± 0.11
		100	1.32 ± 0.28	1.17 ± 0.14	1.13 ± 0.10
	Methanol	1	1.86 ± 0.30	1.53 ± 0.12	1.21 ± 0.08
		10	1.75 ± 0.26	1.65 ± 0.32	1.29 ± 0.08
		100	1.45 ± 0.24	1.18 ± 0.09	1.03 ± 0.06
	Water	1	1.84 ± 0.28	1.38 ± 0.24	0.96 ± 0.06
		10	1.63 ± 0.24	1.24 ± 0.14	1.14 ± 0.09
		100	1.65 ± 0.23	1.26 ± 0.11	1.13 ± 0.08
	Ethnomedicinal	1	1.91 ± 0.31	1.12 ± 0.11	1.14 ± 0.12
		10	1.62 ± 0.28	1.12 ± 0.06	1.13 ± 0.04

Plant name	Extract	Concentration (µg/mL)	Intracellular ROS (fold change) relative to negative control ± SEM		
			C2C12	SC-1	THP-1
		100	1.44 ± 0.24	1.23 ± 0.16	1.24 ± 0.11
Trolox		5	1.34 ± 0.31	1.00 ± 0.09	0.83 ± 0.06
AAPH		100 µM	2.82 ± 0.44	1.70 ± 0.20	1.57 ± 0.26

SEM, standard error of mean

4.5.2.2. *Boerhavia diffusa*

Apart from the water extract, all other extracts of *B. diffusa* decreased intracellular ROS in C2C12 myoblast cells in a concentration-dependent manner (Figure 45, Table 12). The ethnomedicinal extract of the plant produced the most profound effect against ROS generation in the cells, with a 1.81- and 2.33-fold decrease at 1 and 100 µg/mL, respectively, compared to the AAPH controls. The ethyl acetate extract also significantly ($p < 0.05$) reduced AAPH-induced intracellular ROS at 100 µg/mL (2.41-fold decrease), compared to the AAPH controls. The effect produced by these two extracts against AAPH-induced intracellular ROS is indicative of their potential in the management of oxidative stress-induced wounds. Although not significant up to 100 µg/mL, the hexane and methanol extracts of the plant also caused a concentration-dependent decrease in AAPH-induced oxidation in the cells. This suggests that the two extracts could be useful in the management of ROS-induced wounds at concentrations higher than 100 µg/mL. Such high concentrations could easily be achieved in traditional topical preparations. On the contrary, a slight increase in ROS was observed upon pre-treatment with increasing concentrations of the water extract of the plant. Whereas this effect could derail healing of oxidative stress-induced wounds, the extract could aid the healing of other wound types because of a possible role against infecting organisms.[5, 157]

The ethyl acetate, methanol, and water extracts of the plant had no observed effect against AAPH-induced oxidation in SC-1 fibroblasts (Figure 45). This suggests that the three extracts may not have a protective effect in an oxidative stress situation. Moreover, the hexane and ethnomedicinal extracts of the plant were also not observed to suppress ROS release in the fibroblasts. On the contrary, the two extracts caused a concentration-dependent increase in intracellular ROS. Whereas intracellular ROS upon pre-treatment with the 1 µg/mL hexane extract was similar to the AAPH controls, for instance, it was increased by 1.25-fold at 100 µg/mL. These extracts, though not

likely to be useful in protecting the cells against oxidative stress, could decrease chances of wound infection and thereby enhance healing.

All extracts of *B. diffusa*, however, caused a concentration-dependent reduction in AAPH-induced oxidation in the differentiated THP-1 macrophages, howbeit insignificant (Figure 45). Intracellular ROS was reduced from 1.57-fold in the AAPH-controls to 1.20-, 1.08-, 1.04-, 1.19-, and 1.03-fold upon pre-treatment with the hexane, ethyl acetate, methanol, water, and ethnomedicinal extracts of the plant, respectively. These suggest that the extracts could be effective in protection against oxidative stress at concentrations higher than 100 µg/mL. Although such high concentrations could have safety implications systemically, they could be achieved in topical preparations.

The observed effects of the extracts in C2C12 myoblast and THP-1 macrophages could be due to the antioxidant activity of the extracts.[27] With an IC_{50} of 21.23 µg/mL, the ethyl acetate extract of *B. diffusa* had the strongest ABTS radical scavenging activity (Table 11, Figures 42 and 43). The other extracts also recorded moderate effects on the free radicals. Furthermore, various phytochemicals containing antioxidant activity[78, 192, 193] were detected in the extracts (Figures 16 – 17). Quercetin was detected in all, but the ethyl acetate extract; kaempferol in all but the ethyl acetate and water extracts; and rutin in the ethyl acetate and methanol extracts.

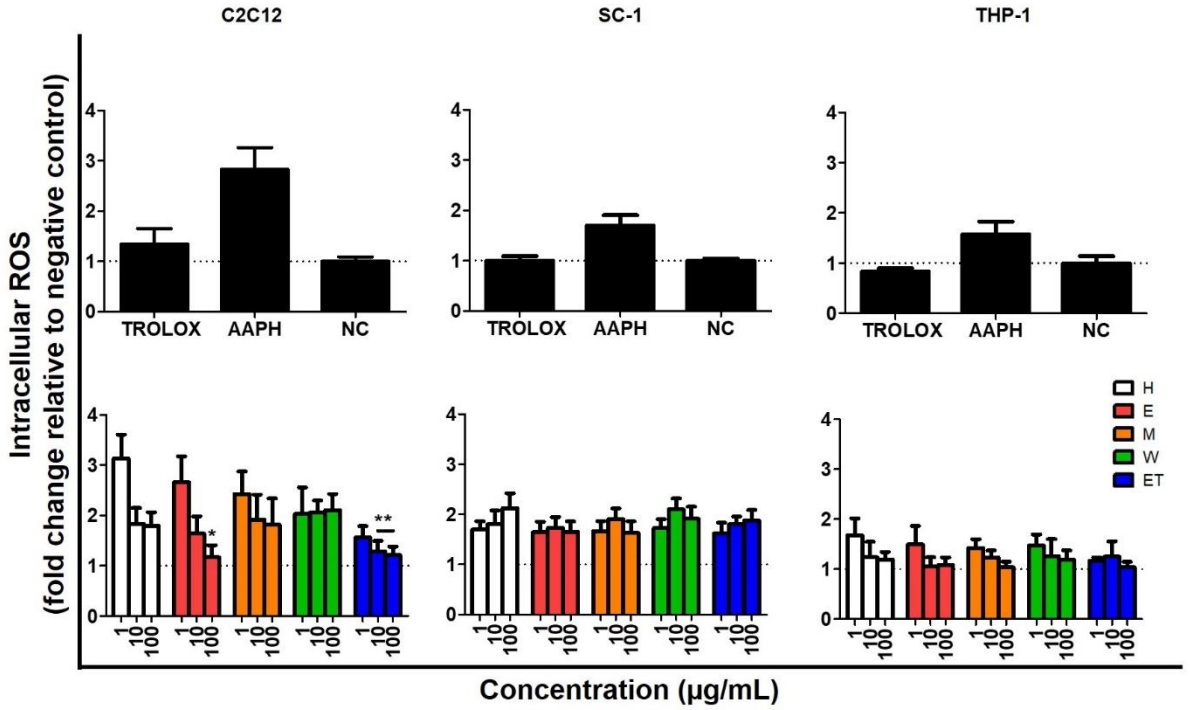

Figure 45: The fold-change in ROS of C2C12 myoblast, SC-1 fibroblast, and THP-1 macrophage cells treated with AAPH after pre-treatment with the hexane (H), ethyl acetate (E), methanol (M), water (W) and ethnomedicinal extracts (ET) of *B. diffusa.* AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride; NC, negative control. Statistical significance relative to AAPH control: $p < 0.05$ (*), $p < 0.01$ (**).

It is not clear yet as what could be responsible for the pro-oxidant effect observed in the fibroblasts when pre-treated with the hexane and ethnomedicinal extracts of *B. diffusa*. Some phytochemical components, including quercetin and kaempferol, have been shown to have pro-oxidant activities at high doses and in the presence of metal ions.[194] Therefore, the observed effect could be due to presence of these compounds in the extracts. Further studies would, however, be required to confirm this.

A study that investigated the antioxidant and genoprotective ability of rotenoids from the roots of *B. diffusa* reported a possible inhibition of ROS formation in human colon adenocarcinoma (Caco-2) cells.[188] Pre-treatment of the cells with boeravinone G, one of the rotenoids, caused a suppression of H_2O_2-induced ROS formation. Another study also reported the inhibition of angiotensin II-induced ROS formation in H9C2 cardiomyoblasts by the ethanolic extract of *B. diffusa*.[195] Both these studies indicate that *B. diffusa* has the potential to decrease intracellular ROS levels.

Although H9C2 cardiomyoblasts are physiologically not entirely similar to C2C12 myoblasts, there are some morphological and functional relationships between the cells. Like the previous account, some of the extracts prepared from the plant in the current study showed potential to inhibit ROS in C2C12 cells, thereby corroborating the findings published previously. No literature could be found on the effect of *B. diffusa* extracts or compounds on intracellular ROS formation in fibroblasts and macrophages.

4.5.2.3. *Erythrina senegalensis*

Most of the extracts of *E. senegalensis* produced a concentration-dependent suppression of AAPH-induced oxidation in the three cell types, albeit generally insignificant (Figure 46, Table 12). All five extracts of the plant marginally decreased intracellular ROS in C2C12 myoblasts, especially at 100 µg/mL. The most prominent effect was observed upon pre-treatment with the ethyl acetate extract of the plant, with a 2.14-fold decrease at 100 µg/mL, compared to the AAPH-controls. All other extracts caused a 1.58- to 1.96-fold decrease in intracellular ROS at 100 µg/mL, when compared to the AAPH controls. This indicates that all extracts of the plant could be useful in protecting myoblasts against oxidative stress, and hence facilitate wound healing.

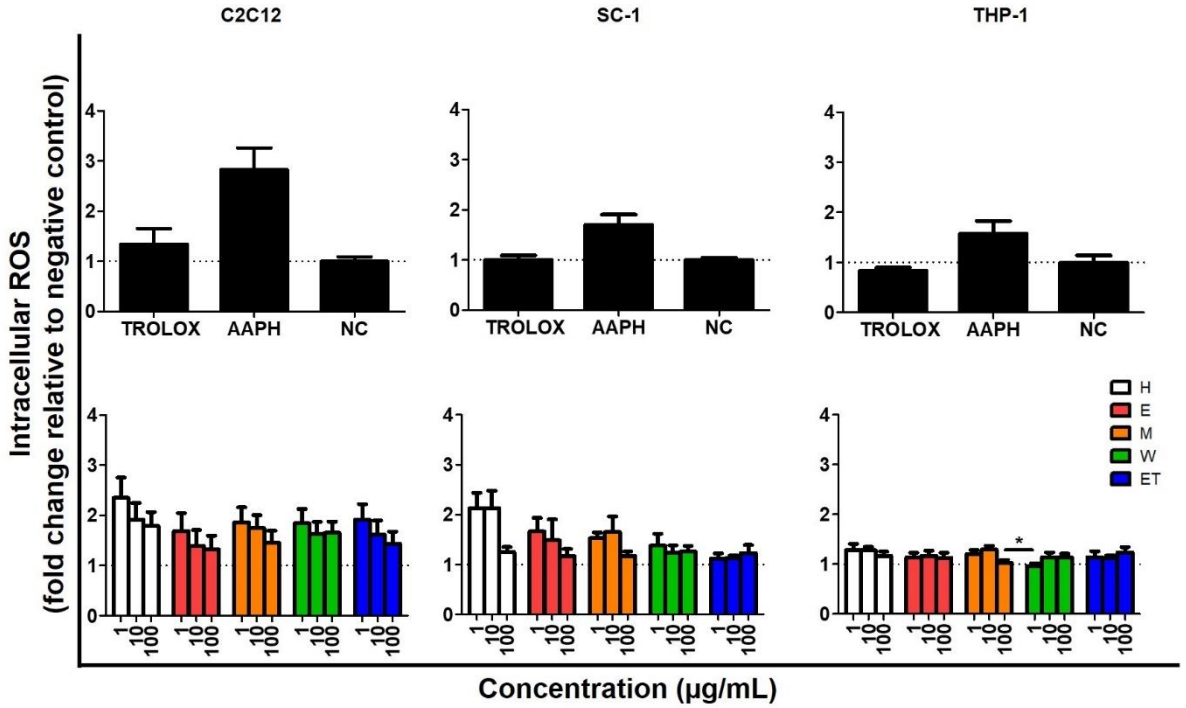

Figure 46: The fold-change in ROS of C2C12 myoblast, SC-1 fibroblast, and THP-1 macrophage cells treated with AAPH after pre-treatment with the hexane (H), ethyl acetate (E), methanol (M), water (W) and ethnomedicinal extracts (ET) of *E. senegalensis.* AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride; NC, negative control. Statistical significance relative to AAPH control: $p < 0.05$ (*), $p < 0.01$ (**)

The extracts, except for the ethnomedicinal, produced a similar result against AAPH-induced oxidation in SC-1 fibroblasts (Figure 46). Pre-treatment of the cells with the hexane, ethyl acetate, methanol, and water extracts of the plant decreased oxidative stress in a concentration-dependent way, with a 1.36-, 1.45-, 1.44-, and 1.35-fold reduction in intracellular ROS, respectively, at 100 µg/mL compared to the AAPH controls. These four extracts could therefore be effective in the management of ROS-induced wounds at concentrations higher than 100 µg/mL. Since ethnomedicinal wound-healing products are often used topically, such concentrations could easily be achieved. The ethnomedicinal extract of the plant exhibited a concentration-dependent pro-oxidant effect. Whilst pre-treatment with 1 µg/mL of the extract decreased ROS release by 1.52-fold when compared to the AAPH treated cells, the 100 µg/mL extract treatment produced a 1.38-fold inhibition. Though this extract could facilitate healing of ROS-induced wound healing at lower concentrations, concentrations above 100 µg/mL could prove detrimental in such wounds. The pro-

oxidant effect exhibited at higher concentrations, however, could make the extract useful in the management of wound infection. Phytochemical analysis did not confirm the presence of any compound with pro-oxidant activity. The extract, however, tested positive for flavonoids and phenolic components (Table 8). Several compounds belonging to these phytochemical classes have been shown to have pro-oxidant effects at higher concentrations.[194] Though kaempferol and quercetin were not detected using UPLC-MS, other pro-oxidant phenolic compounds may be present. The hexane extract of the plant had the least effect on ROS release, inhibiting it by 64.3% at 100 µg/mL.

The extracts strongly inhibited intracellular ROS in THP-1 macrophages (Figure 46). The most significant effect was observed in the cells pre-treated with the methanol extract, where intracellular ROS levels were 1.52-fold lower than in the AAPH-only treated cells. The hexane and ethyl acetate extracts of the plant marginally (maximum of 1.39-fold) decreased intracellular ROS in a concentration-dependent manner. This suggest that the three extracts could enhance healing of ROS-induced wounds by suppressing ROS. The water and ethnomedicinal extracts, however, produced a slight concentration-dependent increase in intracellular ROS. Pre-treatment with the water extract at 1 µg/mL, for instance, decreased ROS by 1.64-fold, compared to 1.39-fold in cells pre-treated with 100 µg/mL of the extract. This pro-oxidant effect exhibited by the extracts at higher concentrations could indicate a possible antimicrobial function at concentrations above 100 µg/mL. This could particularly be useful in the management of non-ROS related wounds.

The observed effects correlate with the antioxidant activity noted for the extracts (Table 11, Figures 42 and 43). Whilst extracts with strong free radical scavenging ability, such as the ethyl acetate, methanol, and water, showed the ability to protect against oxidative stress, this was not the case for those with poor antioxidant activity such as the hexane extract. Some antioxidant compounds were detected in the extracts using UPLC-MS analysis (Figures 18 and 19). Rutin was detected in the ethyl acetate and methanol extracts, whilst kaempferol was found in the methanol extract. These compounds have demonstrated antioxidant activities,[78, 192, 193] and therefore could account for the observed effects. The effects observed in the other extracts could be a result of other phytochemical groups present (Table 8).

Only one study was found which described the effect of *E. senegalensis* on intracellular ROS. With the aim of investigating the cytotoxicity and the modes of action of three isoflavonoids from the plant, it was reported that isoneorautenol increased ROS production in CCRF-CEM leukemia cells.[114] Though the hexane extract used in the current study slightly increased ROS in fibroblasts, it is not known which compound is responsible for the activity.

4.5.3. Xanthine oxidase inhibitory effect

The method used by Liu *et al*.[175] was modified to evaluate the effect of the extracts on XO. The positive control, allopurinol, showed strong activity against the enzyme, resulting in a calculated IC_{50} of 2.1 µg/mL. This is comparable to the reported IC_{50} value for the XO inhibitor[196] and therefore an indication that the method worked.

In contrast to allopurinol, there was no significant inhibition of XO activity by extracts of *A. africana* (Figure 47). Although not significant, the extracts appeared to marginally enhance XO activity, especially at concentrations above 50 µg/mL. This suggests a possible pro-oxidant effect of the extracts. This effect was more prominent in the methanol and water extracts. None of the other extracts of the plant also significantly inhibited activity of the enzyme. No reports on the effect of *A. africana* extracts or compounds on xanthine oxidase activity could be obtained. However, some reports on the anti-inflammatory potential of extracts from the plant have been sighted. The hexane leaf extract of the plant has been shown to exhibit anti-inflammatory activity by inhibiting xylene-induced oedema, suppressing development of egg albumin- and agar-induced paw oedema, and the oedematous response to arthritis induced by formaldehyde.[197] Moreover, a previous account reported an enhanced gastroprotection through reduction of acid output, neutrophil infiltration, and oxidative stress upon treatment of rats with the aqueous extract of the leaves.[198]

The apparent marginal effect exhibited by high concentrations of some of the extracts in the current study suggests possible presence of XO inhibitors. Some antioxidants, including quercetin, are common inhibitors of XO activity.[199] The marginal effect on

the enzyme could therefore be due to the presence of quercetin. This however needs to be ascertained.

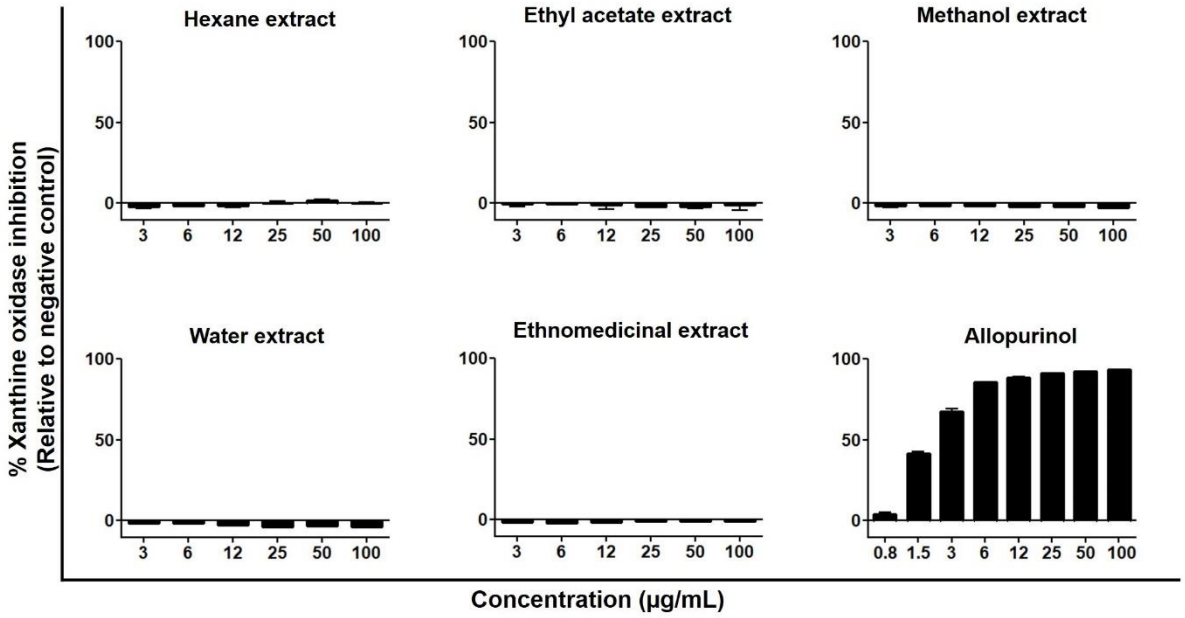

Figure 47: Effect of *A. africana* extracts on xanthine oxidase activity. H, hexane; E, ethyl acetate; M, methanol; W, water; ET, ethnomedicinal; PC, positive control.

The ethyl acetate and methanol extracts of *B. diffusa* also exhibited slight but insignificant XO inhibitory activity (≤5%) even at the highest concentration tested (100 µg/mL) (Figure 48). The hexane, water, and ethyl acetate extracts, however, had no significant inhibitory effects on the enzyme. *B. diffusa* is known to possess diverse anti-inflammatory potential. Ethanol extracts of the plant were previously found to attenuate XO activity in angiotensin II-induced hypertrophic cardiomyoblasts.[195] Also, in an experiment to investigate the immunomodulatory property of the ethanolic extract of the roots, the extract inhibited human NK cell cytotoxicity in the K562 erythroleukemic cell line.[200, 201] It also decreased production of NO in RAW264.7 mouse macrophage cells, as well as IL-2 and TNF-α in human peripheral blood mononuclear cells (PBMCs).[200, 201] An anti-inflammatory rotenoid, boeravinone N, isolated from the methanol root extract strongly inhibited cyclooxygenase 1 (COX-1) and 2 (COX-2) activity, with IC_{50} values being 21.7 and 25.5 µM, respectively.[202] It is unknown why the *B. diffusa* extracts had no significant effect on XO activity in the current study. While differences in sampling sites and extraction procedure could account for this, it is worthwhile to note that most of the previous studies did not report on XO activity. The only study that did, used crude ethanol extracts.[195]

An *in vivo* study indicated that the leaf juice of *B. diffusa*, and the crude water decoction had the ability to inhibit acetic acid-induced abdominal writhing and increase latency in a thermal model of hyperalgesia.[203] Boerhavinone B and a lipid based formulation of a rotenoid-rich fraction prepared from the methanol root extract have also been shown to inhibit carrageenan-induced oedema formation in rats.[202, 204] In a more recent study, the inducible nitric oxide synthase (iNOS) and TNF-α mRNA expression levels were decreased in a mouse gastritis model after treatment with an ethanol leaf extract of *B. diffusa*.[201]

Xanthine oxidase plays a major role in the inflammatory process.[167] Though ethanol extracts of the plant were not employed in the present study, the previous account of Prathapan *et al*.[195] attests to the presence of XO inhibitory compounds in the plant. Interestingly, the ethyl acetate and methanol extracts of *B. diffusa*, which presented with good antioxidant activity, were found to present with some enzyme inhibitory activity. This suggests that the antioxidant compounds quercetin and kaempferol could account for the observed effects. The findings of this study therefore show that XO inhibition could contribute to the anti-inflammatory effects of *B. diffusa*.

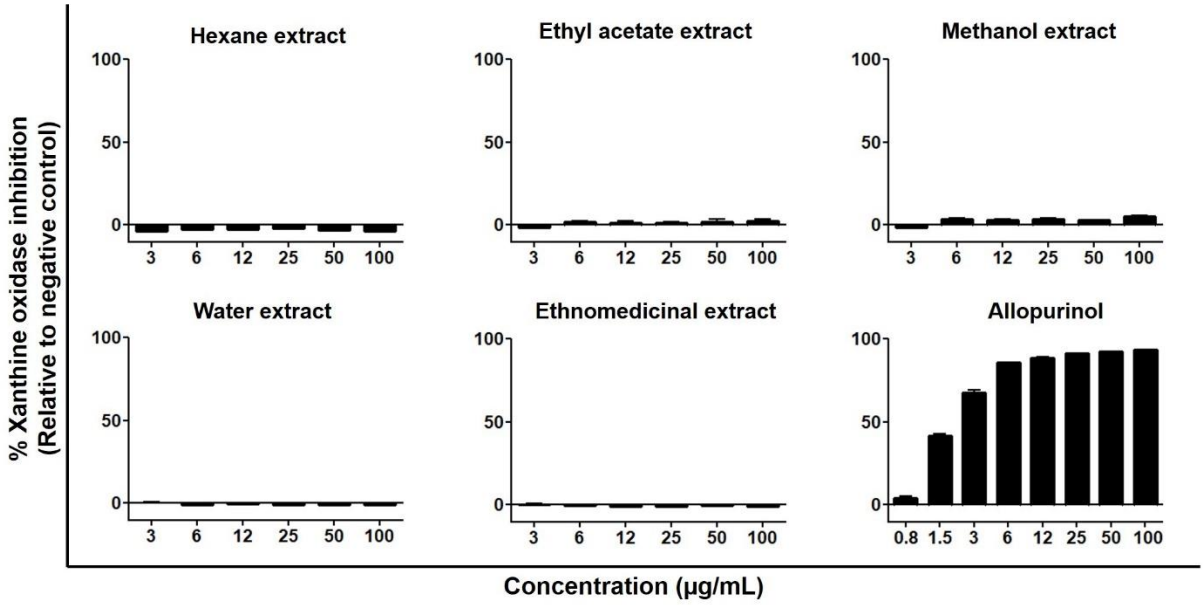

Figure 48: Effect of *B. diffusa* extracts on xanthine oxidase activity. H, hexane; E, ethyl acetate; M, methanol; W, water; ET, ethnomedicinal; PC, positive control.

The ethyl acetate, water, and ethnomedicinal extracts of *E. senegalensis* were found to moderately inhibit XO activity (≤15%), especially at concentrations >50 µg/mL (Figure 49). The hexane and methanol extracts, however, had no observed inhibitory effect on the enzyme's activity. Some previous accounts have illustrated the effect of extracts prepared from the plant on the inflammatory process. Aqueous extracts prepared from the stem-bark of the plant yielded prominent effects in an egg albumin-induced oedema model conducted in rats.[205] Furthermore, two prenylated flavones, sigmoidin A and sigmoidin B, isolated from a related species (*E. sigmoidea*) selectively inhibited 5-lipoxygenase activity, but not COX-1.[206] A pterocarpan (erybraedin F) from the root and stem-bark of *E. senegalensis* was also reported to have a 15-lipoxygenase inhibitory effect.[154] The pterocarpans, biologically active isoflavoids with the ability to act as phytoalexins, have recently been reported as potent anti-inflammatory and antimicrobial compounds.[207]

Although the plant has demonstrated anti-inflammatory activity, no previous account on XO activity could be found. Findings of the present study therefore indicate a possible contribution of XO inhibition to the plant's anti-inflammatory activity. Some antioxidant compounds have been found to be present in the extracts. These could be responsible for the activity observed against the enzyme. Furthermore, neobavaisoflavone, has been reported to inhibit accumulation of nitrite and therefore could have anti-inflammatory effects by suppressing nitric oxide production.[208]

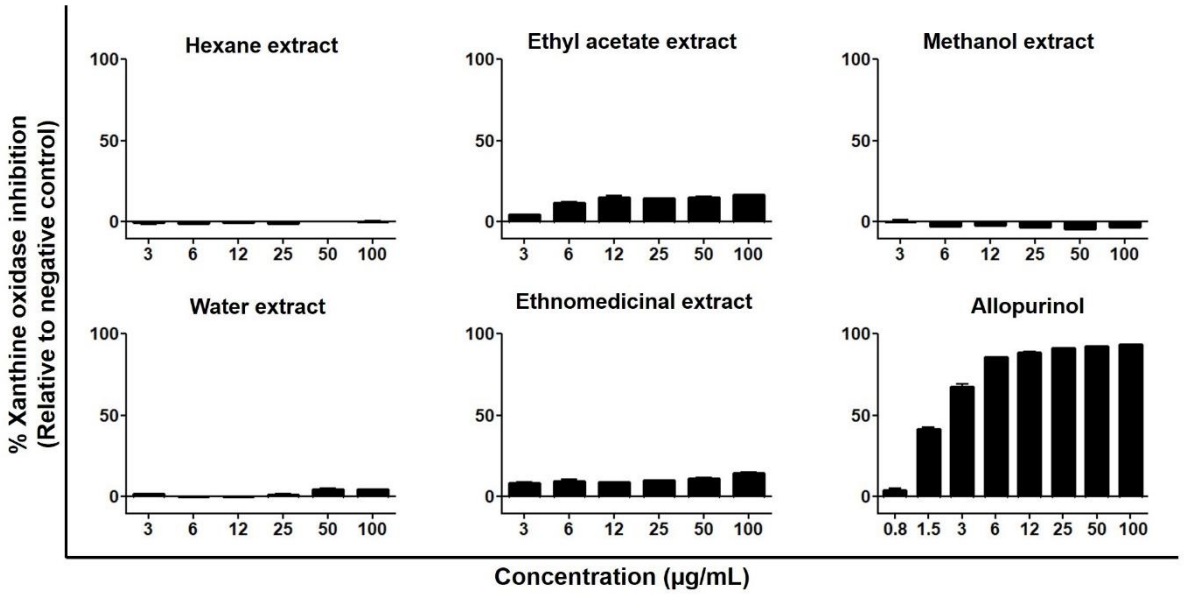

Figure 49: Effect of *E. senegalensis* extracts on xanthine oxidase activity. H, hexane; E, ethyl acetate; M, methanol; W, water; ET, ethnomedicinal; PC, positive control.

4.6. Conclusion

Elevated levels of free radicals and uncontrolled inflammatory response have been linked to the emergence of several clinical conditions, such as chronic wounds. Extracts prepared from the three plants used in this study showed diverse abilities to scavenge the ABTS and DPPH free radical, an indication that they could be useful in the management of conditions involving elevated levels of free radicals, such as oxidative stress-induced chronic wounds. Furthermore, some of the extracts demonstrated the ability to attenuate AAPH-induced oxidative stress in three different mammalian cell lines, confirming their ability to normalize elevated free radicals and their potential in the management of oxidative stress-exacerbated conditions. Though no extracts of *A. africana* showed XO inhibitory activity, some *B. diffusa* and *E. senegalensis* extracts showed minimal reduction of enzyme activity. This suggests a potential anti-inflammatory ability of the extracts through XO inhibition, particularly at higher concentrations. By these findings, the current study has demonstrated that the three plants could be exhibiting their wound healing effects through their antioxidant activity, and ability to inhibit oxidative stress. *B. diffusa* and *E. senegalensis* could, additionally exhibit anti-inflammatory effects.

Chapter 5

Effect of extracts on cellular migration

5.1. Introduction

5.1.1. Cells involved in wound healing

The four stages of wound healing are defined by the appearance and activities of diverse cell types. The role of these cells in wounds is summarized in Table 13. The difference between an acute and chronic wound is the timeous progression of the wound through the various healing phases in the former.[6] Therefore, the presence or absence of the cells involved in the respective phases could determine how long it takes for wounds to heal.

Platelets are the first type of cells to be "called" into action in cases of wounding. These cells contribute to haemostasis through the formation of a thrombus plug and secretion of diverse growth factors, cytokines and matrix proteins (Table 13). The thrombus stops bleeding, establishes a protective barrier, and provides a reservoir for substances secreted by platelet degranulation.[15] The secreted substances from platelets ensure a normal inflammatory process through the activation and chemotaxis of inflammatory cells such as neutrophils and macrophages into the wound.[13] Each of these inflammatory cells is involved in numerous activities aimed at ensuring a sterile wound environment and facilitation of subsequent phases of healing. Whereas neutrophils eliminate invading pathogens by their phagocytic action, macrophages in addition secrete diverse growth factors and cytokines that promote cellular recruitment and activation for subsequent steps in the healing process.[6, 17]

Other cells involved in the healing process include fibroblasts and epithelial cells. Increased activity of fibroblasts characterizes the proliferation phase of wound healing. These cells migrate into the wound from neighbouring tissues in response to the chemotactic action of growth factors mainly secreted by macrophages to proliferate and synthesize collagen, as well as MMPs.[13] Also present in the wound is a class of fibroblasts which transform in response to TGF-β into myofibroblasts for matrix contraction.[15] The proliferation of epithelial cells aids the healing process by promoting the regeneration of damaged blood vessels.[6, 17] Therefore, the restoration

of normalcy to the damaged skin is dependent on the presence and activities of these diverse cellular entities.

Table 13: Role of cells in wound healing (amended from Broughton *et al*.; Enoch and Leaper; Teller and White)[6, 13, 15]

Cell	Wound healing-related function	Phase of wound healing
Platelets	Thrombus formation, secretion of inflammatory mediators (TGF-β, PDGF, PF-4 etc.)	Haemostasis
Neutrophils	Phagocytosis, kills invading bacteria	Inflammation
Monocytes (Macrophages)	Destroys invading pathogens via phagocytosis, secretion of inflammatory mediators (TGF-β, PDGF, EGF, FGF, TNF-α, IL-1, IL-6 etc.), initiates proliferation of fibroblasts, collagen synthesis, and angiogenesis	
Fibroblasts	Synthesize collagen, fibronectin, hyaluronic acid, proteoglycans, forms granulation tissue, organization of provisional ECM	Proliferation Remodelling
Epithelial cells	Angiogenesis, growth factor and cytokine secretion (FGF, TGF-β, TNF-α)	

ECM, extracellular matrix; EGF, epidermal growth factor; FGF, fibroblast growth factor; IL, interleukin; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumour necrosis factor.

5.1.2. Cell migration models

Cell migration, defined as the movement of cells, cell sheets or clusters from one location to the other, is crucial to many biological processes, including angiogenesis, cancer invasion and metastasis, embryogenesis, immunological response, and wound healing.[209] Understanding the mechanism underlying the dynamic behaviour of cells could therefore be of great therapeutic potential. Cell migration can occur in a two-dimensional (2D) or three dimensional (3D) configuration depending on the anatomical and physiological contest.[210] Consequently, available models for assessing migration of cells are either 2D or 3D.

5.1.3. Two-dimensional models

The 2D models are the most commonly used cell migration models because of their simplicity and affordability. These models generally involve cell culturing,

wounding/cell injury, monitoring of the healing process, data acquisition with time-lapse microscopy and data evaluation procedures. These are further sub-classified into cell exclusion/inclusion, and chemotactic assays.[211, 212]

5.1.3.1. Cell exclusion assays

This involves excluding cells from an area of interest within a culture plate or disc either by removal (e.g. scratch assay) or using solid barriers to prevent cell adhesion (stopper-based assay).[212, 213]

5.1.3.1.1. Scratch assay

In this assay, cells are seeded and cultured until they reach a confluent monolayer, after which a mechanical "wound" is generated by scraping with a pipette tip, needle, cotton bud or Teflon spatula.[67] Test samples are added to the well and the wounded area monitored over a period of time. Images of cell movement are captured at regular intervals for estimation of rate of cellular movement into the wound. This is a very useful assay with minimal logistical constrains, enabling real time imaging of cellular movement.[212, 214] However, it is fraught with many limitations. Firstly, the scratch assay has low reproducibility as a result of possible differences in mechanical injury between experiments.[214] To improve reproducibility, care should be taken to ensure uniformity of wounds between experiments. One way of improving uniformity is the use of automated devices such as the silicon-tipped drill press.[215] Also, it is important to have a point of reference within scratches in order to obtain the same field during image acquisition.[67] This could be achieved by using an ultrafine tip marker to create a mark under the wells. The second disadvantage of this assay is the difficulty in distinguishing between cells undergoing proliferation and those migrating in response to chemoattractants. This effect could however be minimised by using low serum concentration in culture, antiproliferative agents such as mitomycin C, and limiting acquisition time to 24 h.[67, 214]

5.1.3.1.2. Stopper-based assays

Stopper-based assays originated from the need to enhance uniformity in wound creation, thereby being devoid of the cellular damage associated with scratch assays. It involves the creation of a cell-free zone within the culture plate or disc and

subsequent assessment of cellular migration into the zone over time.[216] In this assay, a physical barrier consisting of a biocompatible material such as the Teflon fence or silicon is placed onto an area of interest within the plate before seeding, and removed when cells reach confluence. A common example of this assay is the Oris cell migration assay which has been validated for high throughput screening of chemical substances.[217]

Another stopper-based method is the use of liquid stoppers such as agarose gel. A typical example is the agarose drop migration assay described in 1978 by Varani and colleagues.[218] In this approach, a cell suspension is separated from the culture media by first dissolving it in 0.2% (w/v) agarose, followed by cooling in a refrigerator for 10 minutes to allow the agarose to solidify. This traps the cells within a thin agarose membrane. Cell migration out of the agarose drop is then monitored with an inverted microscope. Therefore, unlike the scratch assay, the stopper-based assays have the potential to produce more reproducible results since the “wound” area does not differ much between experiments. However, because cells are not physically damaged as happens in a normal wound, chemoattractants are not released and hence this technique may be more useful for studying conditions which are devoid of direct cellular damage such as cancer metastasis.[67, 219]

5.1.3.2. Chemotactic assays

In 1962, Boyden described a method that uses two compartments separated by a porous membrane to study migration in response to a chemotactic agent.[220] Cells were seeded into the upper compartment, whilst a solution containing the test substance was placed in the lower compartment. The directional cellular movement in response to a gradient created by the chemoattractants was assessed. This assay, also known as the Boyden chamber/Transwell assay, differs from the previous ones to the extent that samples are placed in a separate compartment, and the movement of cells noted over time. Its main advantage over the other migration assays is that both adherent and non-adherent cells can be assessed. A major drawback with the assay is its lack of simplicity and the associated technical challenges.[219] It is also difficult to visualize cells and to obtain time-lapse images of cellular migration. Other chemotactic assays such as the microfluidic chamber and horizontal capillary assays could also be used.[213]

5.1.4. Three-dimensional models

Development of 3D modules became necessary to bridge the gap exiting between *in vitro* experiments and what is actually happening *in vivo*. Though these modules still do not possess all the complexities of the *in vivo* environment, they are more enhanced compared to the 2D modules discussed above. Recent advances have led to the development of skin substitutes for wound research. The human epidermal full-thickness culture derived from human neonatal foreskin tissue is now commercially available. This consists of normal human epidermal keratinocytes and normal human fibroblasts that provides an *in vivo*-like matrix for studying re-epithelialization.[221]

5.2. Aim and objectives of chapter

This chapter was aimed at determining the effect of extracts prepared from the three ethnomedicinal wound healing plants on fibroblast and myoblast migration.

The objectives were to:

- obtain a monolayer culture of cells in 24-well plates.
- determine the ability of the plant extracts to induce cell migration in an artificially generated wound.

5.3. Methodology

5.3.1. Cell culture and maintenance

Cells were cultured and maintained using the method and conditions described in Section 3.3.1. Cells were seeded into 24-well plates at 5×10^4 cells/well 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 to minimize cell proliferation, and incubated for a further 24 h to form a monolayer (Figure 50).

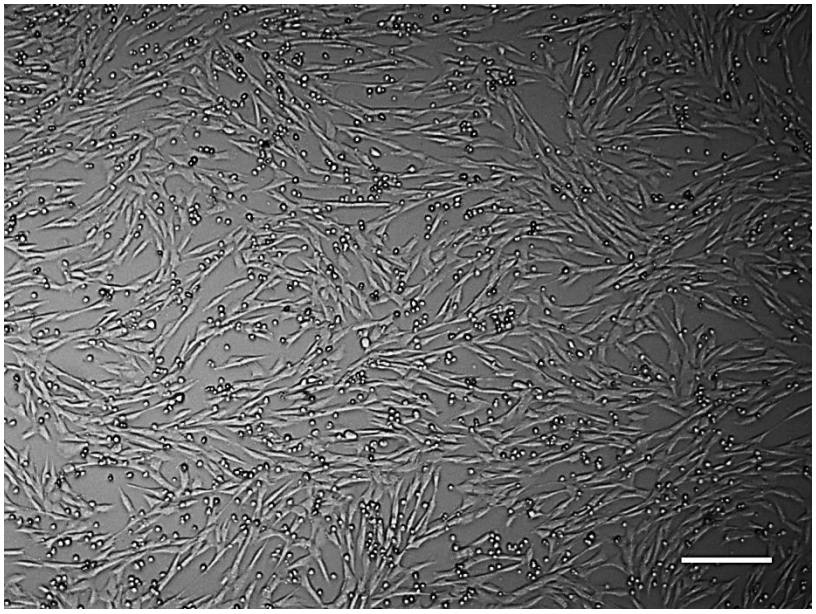

Figure 50: Photomicrograph showing a monolayer of C2C12 myoblast cells through a 5× objective lens. Scale bar = 200 µm

5.3.2. Scratch wound assay

Effect on fibroblast and myoblast cell migration was assessed *in vitro* using the scratch wound assay as previously described.[67] 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 contact is re-established.[67] A sterile micropipette tip was used to stimulate an *in vivo* wound by creating a vertical cell free zone across the cell monolayer in each well. A reference point for image acquisition was generated by creating another cell free zone horizontally to form a cross with the vertical zone. 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.

5.3.3. Data acquisition

Cellular migration was documented by using the snap shot method.[214] Digital photographs of the scratch were captured at 0, 8, and 24 h after wound generation using a bright-field microscope (Axiovert 40 CFL) connected to a ZEISS AxioCam MRm digital camera. The average wound area was estimated using ImageJ software, and cell migration calculated as:

$$Cell\ migration\ (\%\ relative\ to\ negative\ control) = \frac{Ao - At}{Ao} \times 100$$

Where *Ao* and *At* represent average wound area at 0 h and time t respectively.

5.4. Statistical analysis

Data represents results of at least three independent experiments conducted in technical triplicates. Statistical analysis was performed using GraphPad Prism 7 data analysis software. Data was expressed as the mean ± SEM. Analysis of variance (ANOVA) was conducted using the Kruskal-Wallis test followed by Dunn's post-hoc test to determine the difference between two or more groups of data. A *p* value less than 0.05 was considered significant.

5.5. Results and discussion

Different types of cells migrate into the wound to restore structurally damaged tissue. In this study, extracts were evaluated for their ability to attenuate migration of two of the cells involved in the wound healing process, fibroblasts and myoblasts, into an artificially generated wound. The scratch wound healing assay was considered for the study because it is inexpensive and easy to perform, allows observation of cell movement and morphology, and enables easy adjustment of testing conditions.[214] To limit the study to migration and minimize the contribution of proliferation, the serum concentration in culture medium was reduced, and measurements were taken within a period of 24 h.[67]

Cytotoxicity evaluations showed all extracts to have an IC_{50} above 100 µg/mL. Hence, studies on the cellular migratory effect of the extracts was limited to 10 and 100 µg/mL of extracts. The ability of extracts to close the artificially generated wound was varied (Figure 51). Total wound closure in untreated myoblasts and fibroblasts was 56% and 64%, respectively. Exposure of the cells to the positive control, 2 ng/mL PDGF, increased wound closure by an additional 20% and 15% in C2C12 myoblasts and SC-1 fibroblasts, respectively, compared to the negative control.

5.5.1. *Aspilia africana*

The effect of *A. africana* extracts on migration of myoblasts is as shown on Figure 52. All five extracts of *A. africana* enhanced myoblast migration when compared to the negative control, although the effect was mostly minimal (Figure 51A). The most prominent effect was observed after treatment with the hexane extract of the plant. Wound closure of the cells after 24 h treatment with this extract at both concentrations was comparable to the response exhibited by the positive control. Cell migration was increased to 76.0% and 80.5% upon treatment with the 10 µg/mL and 100 µg/mL of the extract, respectively. These were significantly ($p < 0.05$) higher than the 55.7% total migration observed in the negative controls, and similar to the 76.0% increase in migration exhibited by PDGF.

Also, myoblast migration was marginally increased upon treatment with the other extracts, compared to the negative control. The ethyl acetate and water extracts of the plant, at 100 µg/mL, increased migration to 68.1% and 63.7%, respectively. The methanol and ethnomedicinal extracts, however, appeared to have a reduced effect on myoblast migration at higher concentrations. Whereas total myoblast migration upon treatment with the 10 µg/mL methanol extract was 71.0%, it was reduced to 59.8% when concentration was increased to 100 µg/mL. This could be due to the increased stress exhibited on the cells by the extract at higher concentrations. Although the extract was observed to have an IC_{50} above 100 µg/mL, it decreased myoblast cell density significantly ($p < 0.001$, Figure 20). Likewise, treatment with the 10 µg/mL and 100 µg/mL ethnomedicinal extracts of the plant resulted in a total migration of 67.3% and 61.1%, respectively. These observations indicate that the two extracts could alter myoblast function at higher concentrations, although useful at lower concentrations.

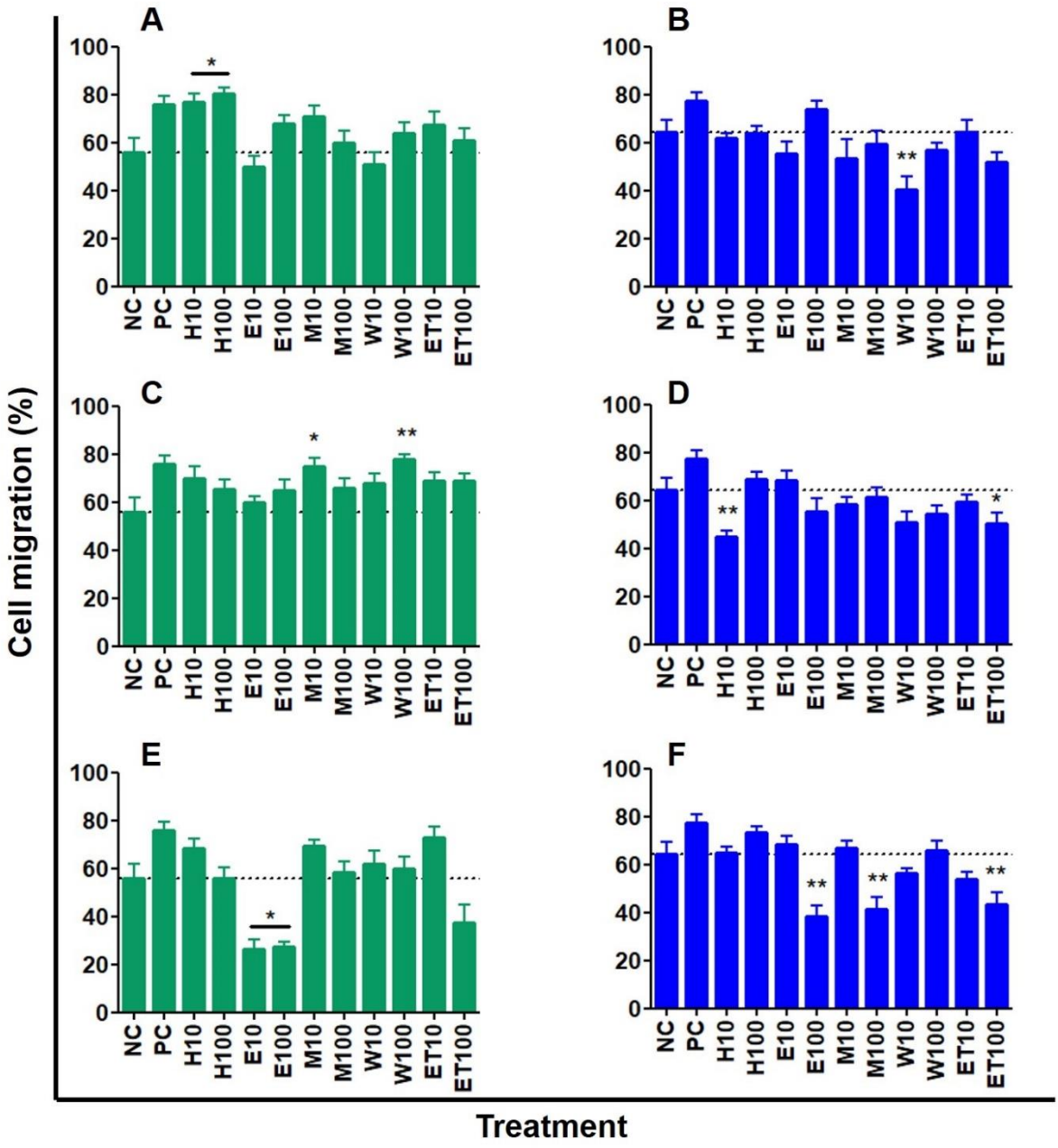

Figure 51: Quantitative determination of C2C12 myoblast (green) and SC-1 fibroblast (blue) cell migration after exposure to extracts prepared from *A. africana* (A, B), *B. diffusa* (C, D), and *E. senegalensis* (E, F) for 24 h. Cells were treated with 10 and 100 µg/mL of the hexane (H), ethyl acetate (E), methanol (M), water (W) and ethnomedicinal (ET) extracts of the plants. NC, negative control; PC, positive control. The dotted line represents maximum cell migration in negative controls. Statistical significance relative to the negative control: $p < 0.05$ (*), $p < 0.01$ (**).

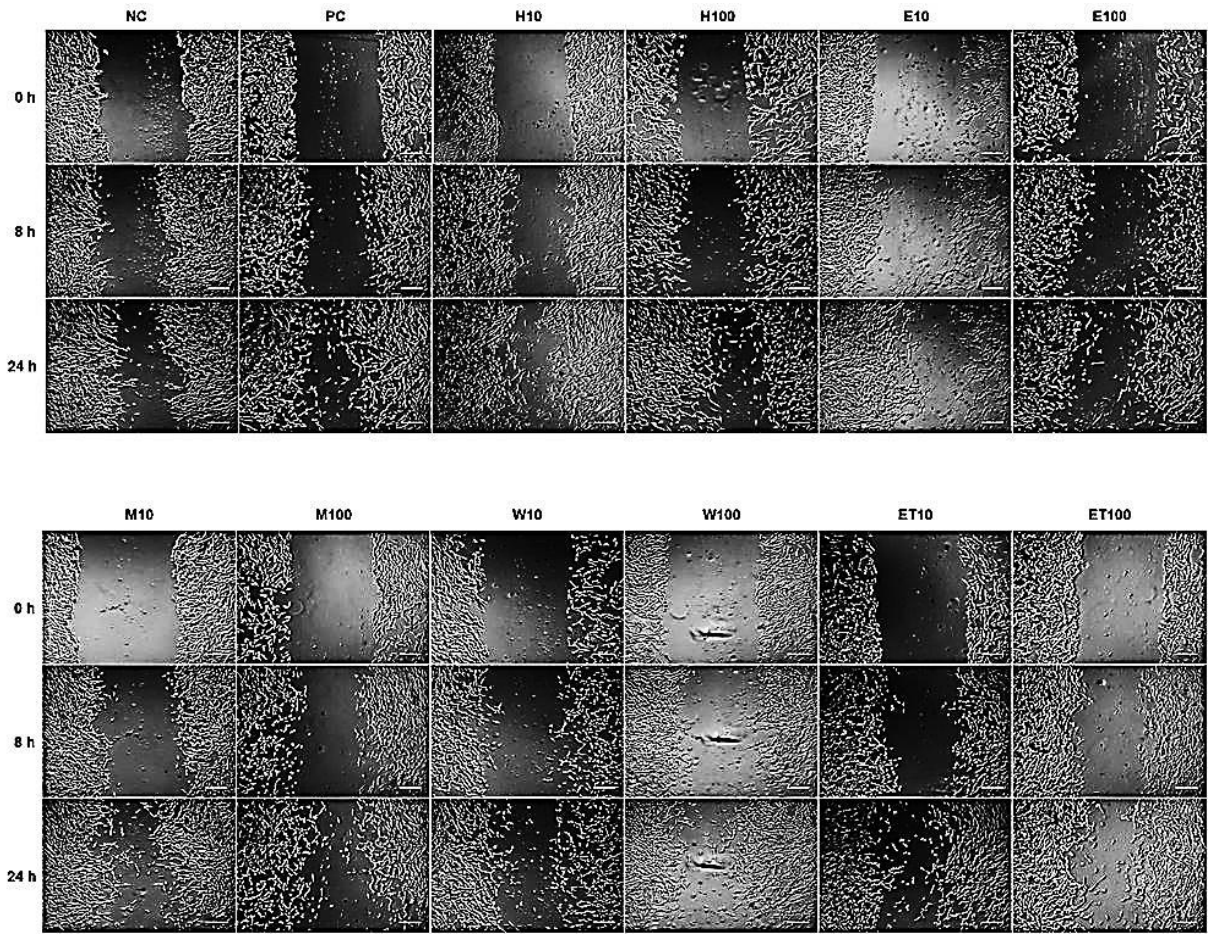

Figure 52: Photomicrographs indicating extent of cellular migration in C2C12 myoblast cells treated with *A. africana* extracts. Cells were treated with the hexane (H), ethyl acetate (E), methanol (M), water (W) and ethnomedicinal (ET) extracts of the plant. NC, negative control; PC, positive control. Images were captured at 0 h, 8 h and 24 h after treatment at 5× magnification. Scale bar = 200 µm.

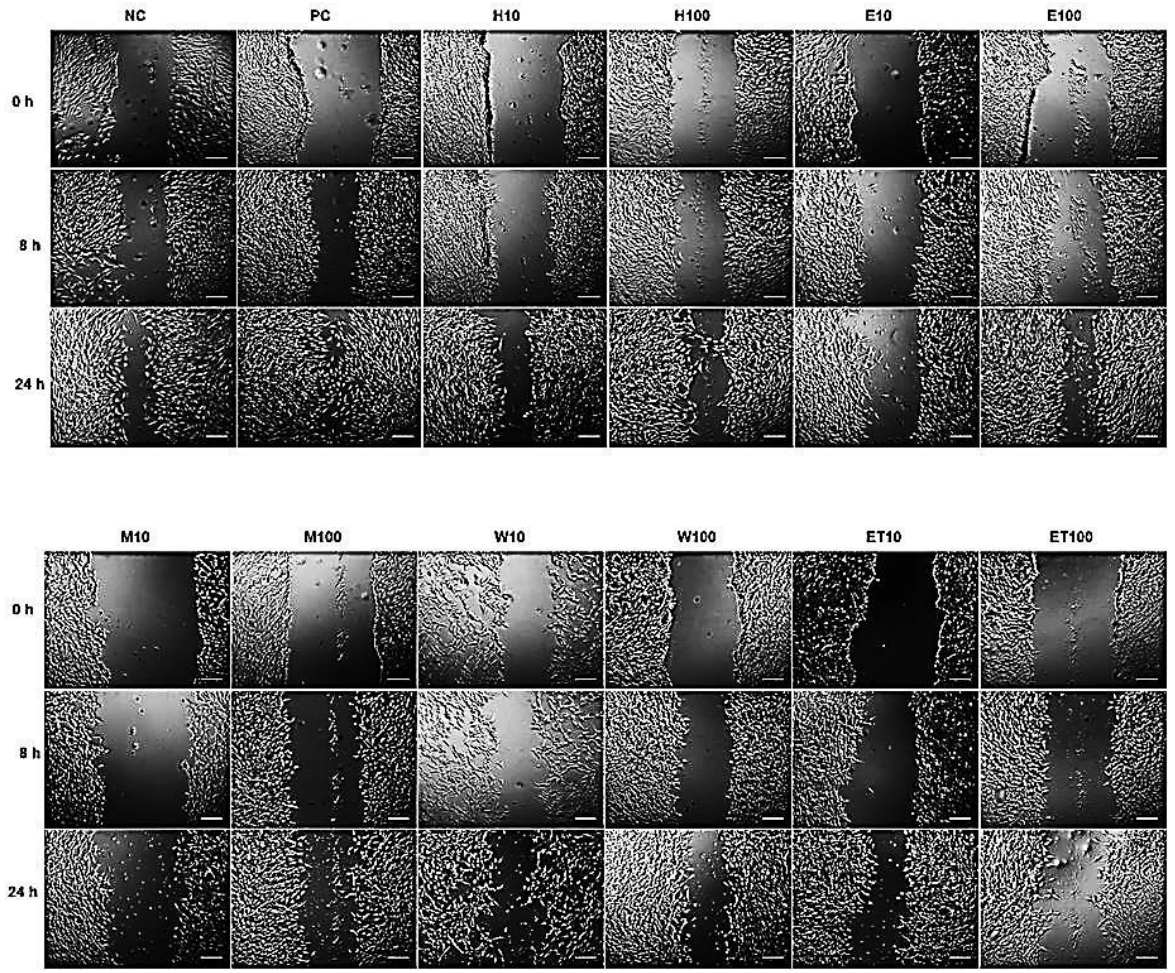

Figure 53: Photomicrographs indicating extent of cellular migration in SC-1 fibroblast cells treated with *A. africana* extracts. Cells were treated with the hexane (H), ethyl acetate (E), methanol (M), water (W) and ethnomedicinal (ET) extracts of the plant. NC, negative control; PC, positive control. Images were captured at 0 h, 8 h and 24 h after treatment at 5× magnification. Scale bar = 200 µm.

The effect of the extracts on SC-1 fibroblast migration was generally more pronounced at higher concentrations, except in the ethnomedicinal extract treatment (Figures 51B, 53). Compared to the negative controls, migration of the cells was decreased to 61.7% and 63.7% upon treatment with the 1 µg/mL and 100 µg/mL hexane extract, respectively. Whilst treatment with the 100 µg/mL ethyl acetate extract resulted in a 14.5% increase in migration, it was similar to the negative controls at 10 µg/mL. The response exhibited by this extract suggests that it could prominently increase fibroblast migration at concentrations higher than 100 µg/mL, thereby enhancing the function of the cells and facilitating healing. Although migration upon treatment with the methanol and water extracts of the plant was generally lower than the negative controls at both concentrations, it was least at 10 µg/mL. On the contrary, whereas migration of the cells was unaltered at 10 µg/mL of the ethnomedicinal extract, it was 19.5% less at 100 µg/mL. This suggest that the extract has potential to impair fibroblast activity at much higher concentrations, and could delay healing.

Scientific literature is scarce with regards to the effect of *A. africana* on cell migration. Whilst no reference to *in vitro* studies could be obtained, two *in vivo* studies were encountered. Intraperitoneal administration of 200 mg/kg of the hexane extract of the plant was reported to exhibit anti-inflammatory activity by inhibiting neutrophil migration and enhancing that of lymphocytes in rats.[197] In a related study, pre-treatment with 500 mg/kg of the aqueous leaf extract of the plant was also shown to inhibit neutrophil migration in indomethacin-induced gastric ulcer rats.[198] Though these two studies are not directly related to the current study, they provide some confirmation that the plant has an effect on cellular migration.

Biological activity of medicinal plants is largely due to the presence of secondary metabolites. Phytochemical fingerprinting of extracts indicated the presence of antioxidants, specifically quercetin. There have been conflicting reports on the effect of antioxidants on cell migration. In a previous study it was indicated that cell migration was promoted in murine embryonic fibroblasts by oxidation of Akt2 kinase, suggesting that molecules that have oxidising capability such as ROS could enhance the migration of cells.[222] However, in another report, cathelicidin-OA1, an antioxidant peptide, was found to accelerate migration of human keratinocytes and fibroblasts.[223]

Therefore, it appears as if the effect exhibited by antioxidants on cell migration is both antioxidant and cell dependent.

The most prominent effect on cellular migration observed in this study using *A. africana* was obtained upon treatment of myoblasts with the hexane extract. The extract promoted myoblast migration, whilst producing a null effect on fibroblast migration. It is not yet clear why the extract exhibited different effects in the two cell types despite their similarity. This could be due to the possible long-term cytotoxicity exhibited by the extract in fibroblasts (Figure 20). Whereas myoblast cell density was unaltered after 24 h and 48 h exposure to the extract, fibroblast cell density was 9.2% lesser after 48 h. Therefore, the inability of the extract to enhance migration of fibroblasts could be ascribed to the increased stress exhibited by the extract over time.

5.5.2. *Boerhavia diffusa*

The effect of *B. diffusa* extract treatment on C2C12 myoblast wound closure is as depicted in Figure 54. All the extracts of the plant enhanced cellular migration, with the most prominent effect being observed for the water extract of the plant. This extract increased myoblast migration to 68.0.0% and 77.7% at 10 µg/mL and 100 µg/mL, respectively, compared to the 55.7% recorded in the negative control (Figure 51C). Migration was also higher following treatment with the 100 µg/mL ethyl acetate extract (16.8%), compared to the 10 µg/mL (7.2%). A reduced effect on myoblast migration was observed with increased concentrations of the hexane and the methanol extracts. At 10 µg/mL, the hexane extract increased migration by 25.7%, compared to the negative control. However, migration following treatment with the 100 µg/mL of the extract was noted to be 33.1% less than observed in the 10 µg/mL treatment. A similar result was recorded upon treatment with the methanol extract of the plant, with total migration after 24 h exposure being 75.0% and 65.7% for the 10 µg/mL and 100 µg/mL, respectively. The findings observed for these two extracts suggests that they could impair myoblast activity when administered at higher concentrations.

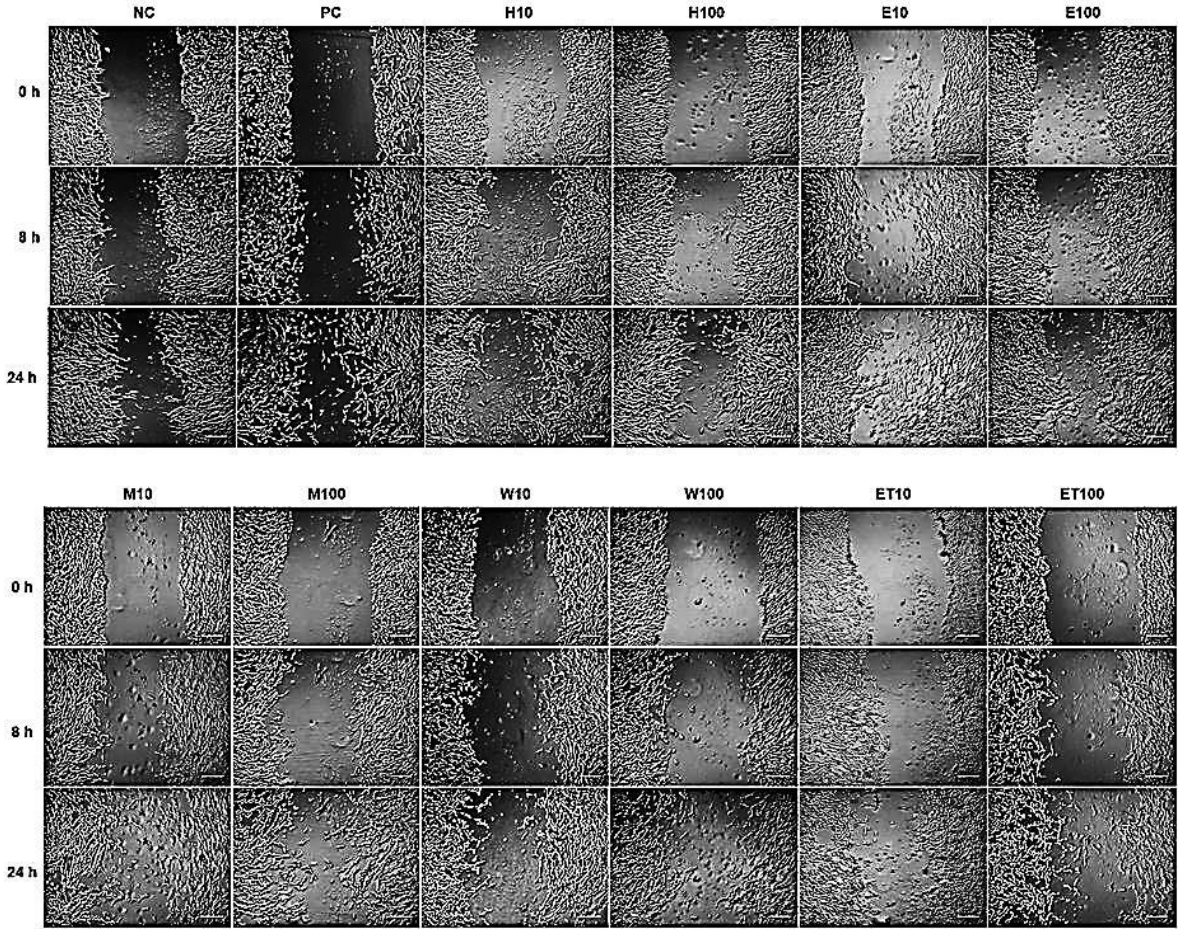

Figure 54: Photomicrographs indicating the extent of cellular migration in C2C12 myoblast cells treated with *B. diffusa* extracts. Cells were treated with the hexane (H), ethyl acetate (E), methanol (M), water (W) and ethnomedicinal (ET) extracts of the plant. NC, negative control; PC, positive control. Images were captured at 0 h, 8 h and 24 h after treatment using 5× magnification. Scale bar = 200 µm.

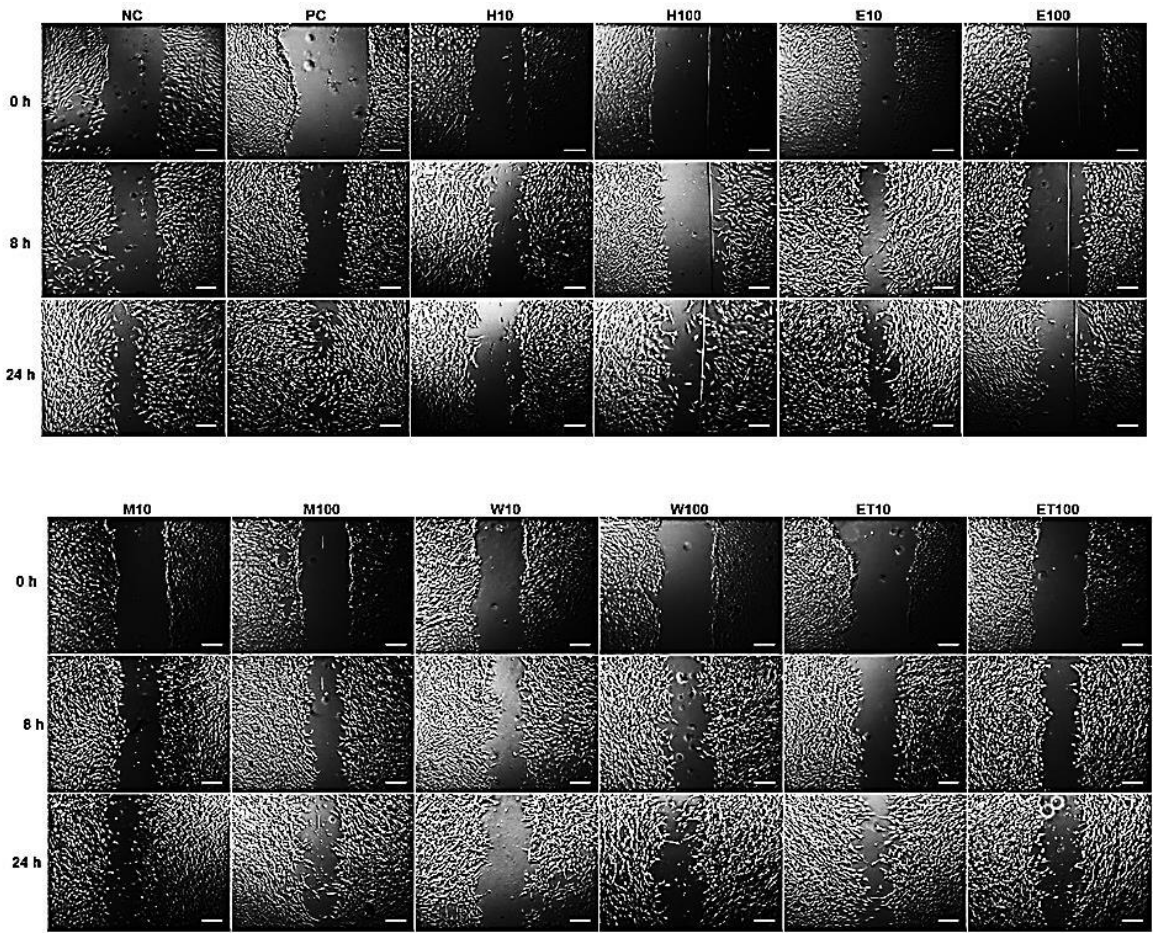

Figure 55: Photomicrographs indicating the extent of cellular migration in SC-1 fibroblast cells treated with *B. diffusa* extracts. Cells were treated with the hexane (H), ethyl acetate (E), methanol (M), water (W) and ethnomedicinal (ET) extracts of the plant. NC, negative control; PC, positive control. Images were captured at 0 h, 8 h and 24 h after treatment using 5× magnification. Scale bar = 200 µm.

The ethnomedicinal extract of the plant, however, recorded a similar effect on migration of the cells at both treatment levels. Whereas the 10 µg/mL of the extract increased migration by 24.0% when compared to the negative controls, the result following treatment with 100 µg/mL was only 2.1% less than the former. The enhanced effect of the extracts on migration of the cells indicate that facilitation of the cells activity could be one of the plant's mechanisms of wound healing.

Most of the extracts had no significant effect on wound closure in the SC-1 fibroblasts, when compared to the negative control (Figure 55). Cell migration was marginally enhanced upon treatment with the hexane and ethyl acetate extracts of the plant at 100 µg/mL and 10 µg/mL, respectively (Figure 51D). However, 10 µg/mL of the former decreased migration by 44.8%, compared to the 64.4% observed in the negative control. A similar, but less pronounced (21.8% decrease) effect was observed upon treatment of the cells with the 100 µg/mL ethnomedicinal extract. Whilst the latter could possibly be attributed to activity of some inhibitors of migration, that of the hexane extract suggests presence of both inhibitors and stimulants of cell migration. This was the case in a study on the radical scavenging activity of curcumin.[224] Whilst a good scavenger of hydroxyl radical at high concentration, it activates the Fenton system to generate an increased amount of hydroxyl radical at lower concentrations.[224] Further studies will be required to ascertain this effect.

Despite the huge anecdotal evidence of the use of *B. diffusa* in wound management,[78, 81, 193] not much records on the scientific evaluation of its *in vitro* effects on wound closure were obtained. Most of the literature is focused on the anti-metastatic activity of the plant. The aqueous extract of the plant was reported to reduce pulmonary metastasis in B16F-10 melanoma cells.[225] Not long afterwards, there was another report of the ability of 70% ethanolic extract of the plant to downregulate granulocyte macrophage-colony stimulating factor (GM-CSF), a known enhancer of cell migration, also in B16F-10 melanoma cells.[226] This activity has been attributed to the alkaloid, punarnavine.[148, 227, 228]

Whilst all these published accounts provide evidence to indicate the presence of inhibitors of cell migration, there are also numerous accounts which indicate the presence of potent antioxidants in the plant.[78] The findings of these previous accounts

have also been corroborated by the current study, with detection of various antioxidant groups and compounds. These antioxidants, together with other unidentified compounds in the plant, have the potential to stimulate wound healing by enhancing cell migration.[222] Therefore, it can be deduced from the findings that the extracts may possess both anti-metastatic and cell migration enhancing compounds. Such migration-enhancing compounds could partly be responsible for the effects observed upon treatment of myoblasts with the methanol and water extracts of the plant.

5.5.3. *Erythrina senegalensis*

The effect of *E. senegalensis* extracts on myoblast migration is indicated in Figures 51E and 56. Apart from the ethyl acetate extract of the plant, none of the other extracts had a major effect on myoblast migration. Whereas migration of the cells was generally increased marginally by the other extracts, the ethyl acetate extract significantly decreased migration ($p < 0.05$). The extract decreased myoblast migration by half at both treatment concentrations after exposure for 24 h, compared to the negative control. A similar effect was exhibited by the ethnomedicinal extract of the plant. Although not significantly different from the negative control, cellular migration in myoblasts treated with the 100 µg/mL ethnomedicinal extract was decreased to 37.2%, compared to 55.7% in the negative control cells. However, the extract at 10 µg/mL increased migration to 73.1%. Moreover, treatment of the cells with the hexane, methanol, and water extracts of the plant also resulted in a better enhanced effect on migration at 10 µg/mL. This may be ascribed to the presence of compounds with the ability to inhibit myoblast migration.[222] Therefore use of extracts from this plant at higher concentrations could hinder myoblast migration, and thus limit the healing process.

The hexane and water extracts, did not have a significant effect on fibroblast migration, although treatment with the 100 µg/mL of the extract increased it marginally, in comparison to the negative control (Figures 51F and 57). The ethyl acetate, methanol, and ethnomedicinal extracts of the plant, however, inhibited migration of the cells. Fibroblast migration was inhibited by 40%, 36%, and 32%, respectively, upon treatment with 100 µg/mL of the extracts for 24 h. Although this effect could be ascribed to the presence of inhibitors of cellular migration, it could also be a result of cellular stress. All three extracts were observed to be negligibly cytotoxic in fibroblasts

(Figure 34). This could have contributed to the diminished cellular migration observed. Furthermore, previous studies[114, 229] as well as the present study identified the presence of cytotoxic isoflavonoides which includes neobavaisoflavone in the plant (Figures 18 and 19). Moreover, another cytotoxic compound (rotenone) was, in addition, detected in the methanol extract of the plant (Figure 19). Presence of this compound could partly explain the diminished effect of the extract against fibroblast migration at higher concentrations because of its ability to induce oxidative stress and apoptosis. [152, 153]

The usefulness of *E. senegalensis* as remedy for wounds in ethnomedicine is widely known.[230] However, not much scientific information is available to support its traditional use. The only study encountered was that of Ilodigwe *et al*. where the excision wound model was employed in albino rats.[79] In their account, topical application of an ointment formulated from the ethanol extract of the plant produced a significant increase in wound contraction and epithelialization, two processes that are dependent on cellular migration.[13, 15] This study, though not similar to the current work, is an indication of the ability of extracts prepared from the plant to attenuate cell migration. All the extracts used in the current study, except the ethyl acetate extract, marginally enhanced myoblast migration. This could be useful for collagen deposition and wound remodelling.[13, 15] Moreover, the hexane and water extracts, at 100 µg/mL, demonstrated the ability to stimulate fibroblast migration. Enhanced fibroblast activity could promote collagen synthesis and wound contraction.[13] This suggests that the increased effect of the plant on wound contraction reported earlier could be a result of enhanced fibroblast migration.[79]

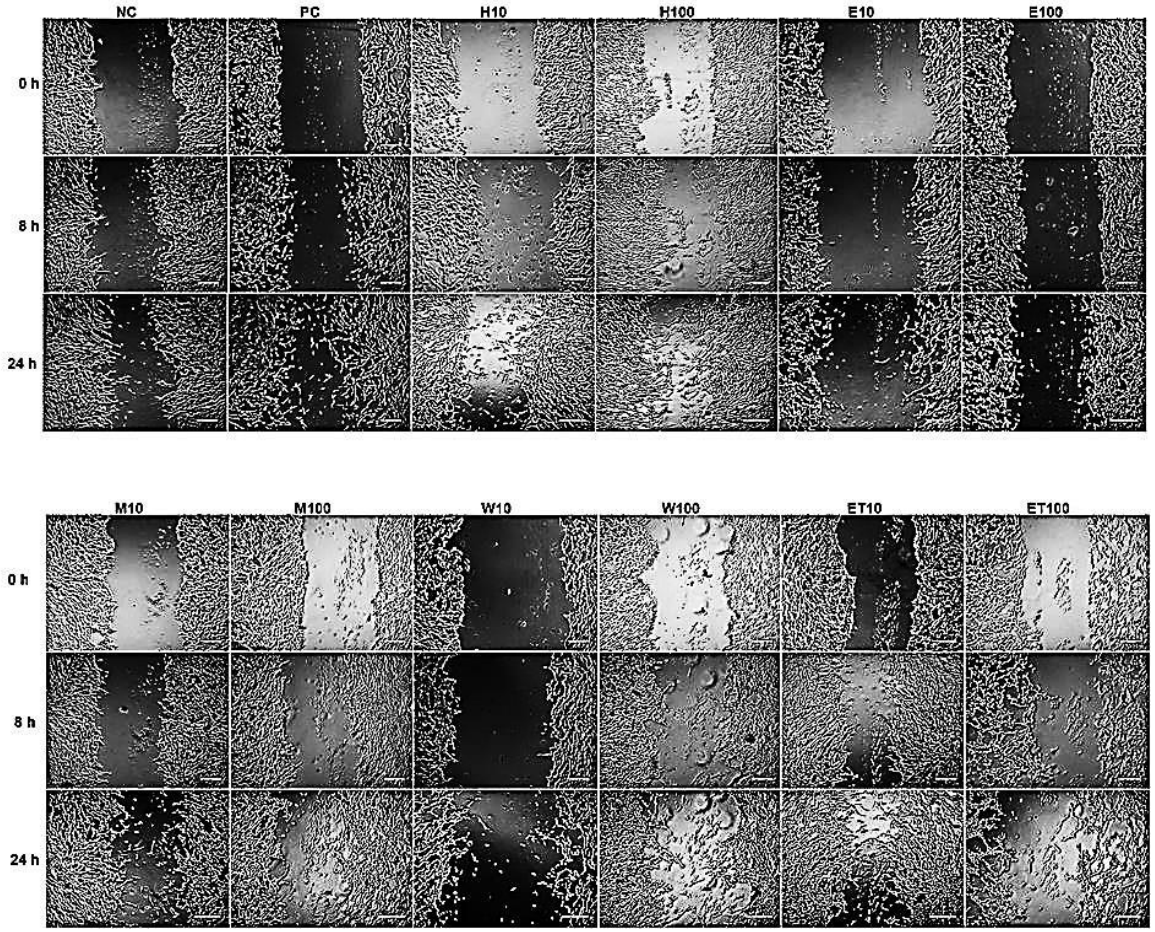

Figure 56: Photomicrographs indicating the extent of cellular migration in C2C12 myoblast cells treated with *E. senegalensis* extracts. Cells were treated with the hexane (H), ethyl acetate (E), methanol (M), water (W) and ethnomedicinal (ET) extracts of the plant. NC, negative control; PC, positive control. Images were captured at 0 h, 8 h and 24 h after treatment at 5× magnification. Scale bar = 200 µm.

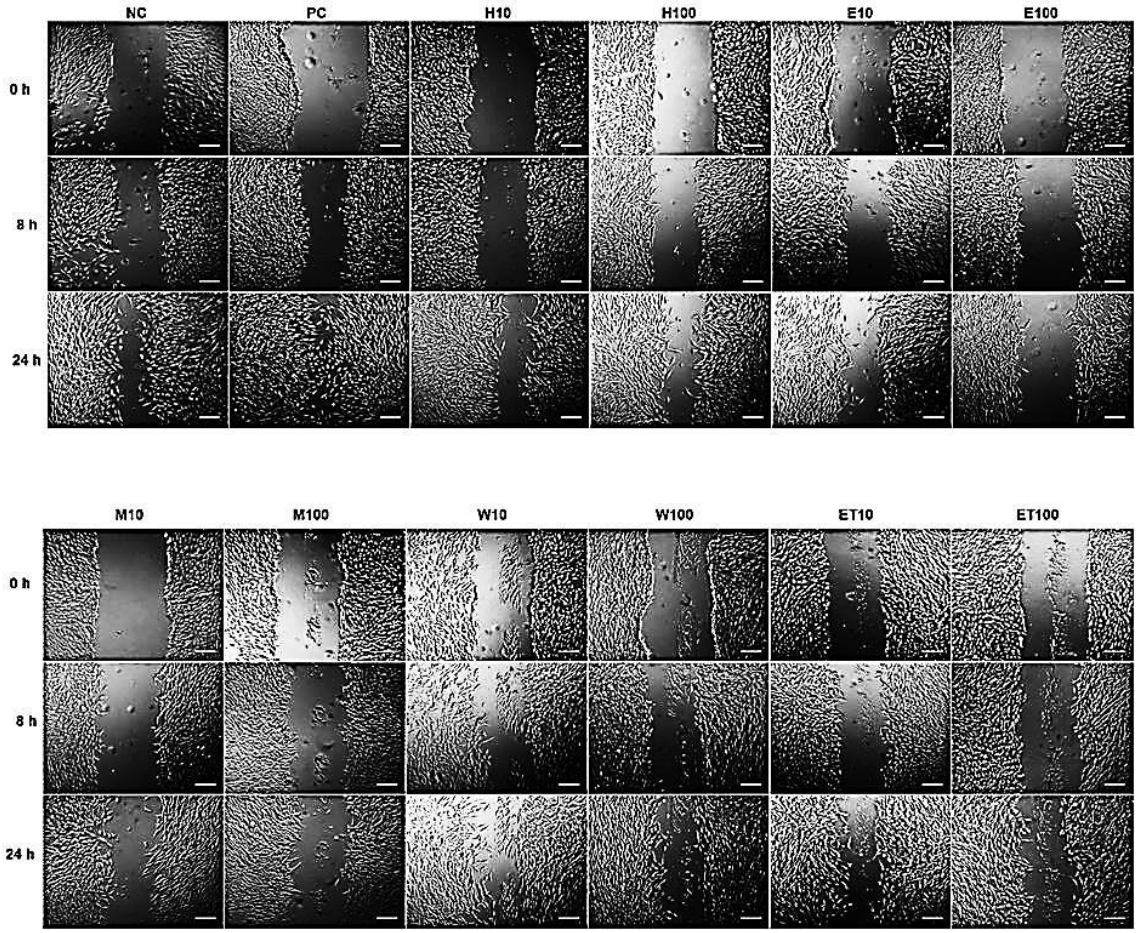

Figure 57: Photomicrographs indicating the extent of cellular migration in SC-1 fibroblast cells treated with *E. senegalensis* extracts. Cells were treated with the hexane (H), ethyl acetate (E), methanol (M), water (W) and ethnomedicinal (ET) extracts of the plant. NC, negative control; PC, positive control. Images were captured at 0 h, 8 h and 24 h after treatment at 5× magnification. Scale bar = 200 µm.

5.6. Conclusion

The scratch wound healing assay was employed to examine the effect of the extracts on myoblast and fibroblast migration. Responses to the extracts were cell line specific. Myoblast migration was enhanced by treatment with the hexane extract of *A. africana*, as well as the methanol and water extracts of *B. diffusa*. This suggests the possible role of the extracts in collagen deposition and wound remodelling, processes that are controlled by myoblasts. Most of the extracts from the three plants marginally increased fibroblast migration. This could also boost wound healing since fibroblasts play a large role in the healing process through activities such as collagen, and MMP synthesis. Some of the extracts, including the ethyl acetate extract of *E. senegalensis*, inhibited cellular migration. This effect could delay wound healing, hence extracts from the plant should be used cautiously. Though the possibility of cytotoxic components contributing to the slower migration of treated cells exists, it could also be due to the presence of migratory inhibitors.

Chapter 6

Antimicrobial effect of extracts

6.1. Introduction

6.1.1. Impact of infection on wound chronicity

Bacterial infection has been reported as one of the common compounding factors that contribute to delays in wound healing.[231] Since wounds have generally lost the protection that an intact epithelial covering provides, bacterial colonization is common.[5] This could overwhelm cellular defence structures, increasing the chances of wounds becoming infected. One of the goals of the inflammatory phase of the wound healing process is to rid the wound of all unwanted substances, including bacteria. As a result, the activation, migration and proliferation of cells such as neutrophils, fibroblasts and endothelial cells required for the timeous healing of wounds occurs.[5] By so doing, the wound healing process takes place without restriction. The opposite is, however, true for chronic wounds.

Colonization of the wound by bacteria could alter the inflammatory process and result in extending the healing time. The presence of bacteria attracts inflammatory cells such as neutrophils into the wound. These cells eliminate the bacteria using diverse mechanisms including phagocytosis, release of ROS, RNS, and proteases like MMPs.[232] When bacterial load increases, inflammatory activity also increases, causing a prolonged elevation of the levels of pro-inflammatory cytokines, proteases and other factors like ROS.[18, 157] These have the potential to induce damage to healthy body cells. The inflammatory process is thus extended until the removal of the contaminating organism/s is complete. This has negative consequences on other aspects of the healing process such as granulation tissue formation, angiogenesis, epithelialization, collagen formation, and wound contraction.[231, 232] As a result, increased bacterial load has the tendency to enhance the chances of a wound entering into a state of chronicity and failing to heal on time.[231, 232]

The skin and mucous membranes of humans commonly harbour both pathogenic and non-pathogenic microorganisms.[233] These include normal microbial flora such as diphtheroid bacilli (e.g., *Corynebacterium*, *Propionibacterium*), non-haemolytic aerobic

and anaerobic staphylococci (*S. epidermidis* and other coagulase-negative staphylococci, occasionally *S. aureus*, and *Peptostreptococcus species*). Also present are Gram-positive, aerobic, spore-forming bacilli, α-haemolytic streptococci (*viridians streptococci*) and *Enterococci*, and Gram-negative coliform bacilli and *Acinetobacter*. Because of the intact epithelial covering, however, these bacteria are almost harmless to the human body.[233]

However, bacteria gain access to the inner tissues of the skin when wounded, and by so doing produce several virulence factors that have potential to impair healing and promote wound chronicity. The presence of various bacterial species in wounds is associated with delayed healing. Organisms such as *S. aureus*, *Pseudomonas aeruginosa*, and β-haemolytic streptococci at levels above 10^5/g in wounds play a central role in delaying healing.[234] This is achieved through the production of tissue destroying enzymes, antiphagocytic and adherence mechanisms, as well as endo- and exotoxins.[234].

6.1.2. Biofilms and resistance to treatment

The discovery of antibiotics for the treatment of bacterial infections was regarded as a breakthrough in modern science, leading to a dramatic decrease in mortality.[235] However, barely four years after the introduction of antibiotics, resistant strains of bacteria emerged due to the inappropriate use thereof. Since then, resistance to several antibiotics has caused an increase in morbidity and mortality of affected patients.[236] This has invariably increased the cost of treatment, as a combination of drugs, and in some cases a longer period of time is often required to treat the infection.[235, 236]

Common mechanisms by which bacteria develop resistance include enzymatic inactivation, target site alteration, acquisition of alternative metabolic pathways and efflux pumps.[235, 236] However, increasing evidence suggests that one of the ways in which bacteria evade the effect of antibiotics, especially on solid surfaces, is through the formation of biofilms.[235, 237] These are a consortia of planktonic cells embedded in a self-produced extracellular polysaccharide matrix (EPS). The biofilm formation process can be summarized as (1) production of a conditioning film consisting of

micro/macromolecules like glycoproteins, (2) planktonic cell transport to the surface, (3) adhesion of microbial cells to conditioned surface, (4) microcolony and EPS formation, and (5) growth and detachment (Figure 58).[235] Within the biofilm, bacteria change their phenotypes, resulting in altered production of virulence factors in response to signalling molecules secreted by other bacteria within the EPS.[238] They also have a more sessile growth and slower metabolic rate.[238] As a result, biofilms are more resistant to antibiotic treatment, host defence mechanisms and environmental stress conditions.

When established within a wound, the physical barrier that biofilms create inhibits re-epithelialization, and the endotoxin released by the bacteria induces a chronic inflammatory response that interferes with the healing process.[34, 238] The characterization of the microorganisms inhabiting chronic and acute wounds of clinical patients revealed the presence of densely aggregated colonies of bacteria often surrounded by an extracellular matrix in delayed wounds.[237] This indicates presence of biofilm forms of bacteria in the wounds. Several other studies attest to the significant contribution of biofilms to wound healing delays.[239] Consequently, many treatment options have been rendered inactive, with an urgent call for intensive research into the identification of more effective alternative antibiofilm agents.[34, 235, 239]

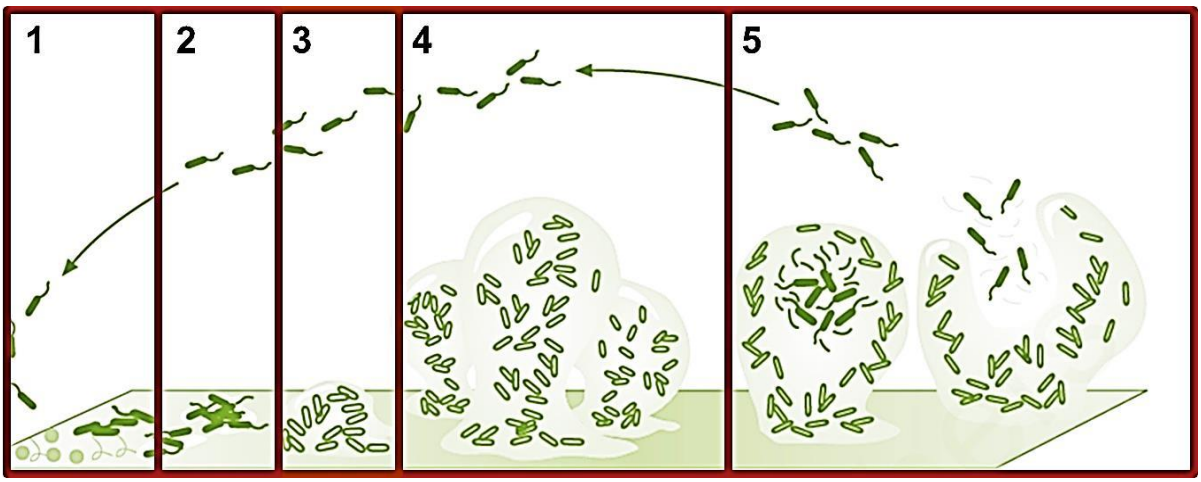

Figure 58: Stages of biofilm formation. Adapted from Stoodley *et al*. [240] 1, Production of a conditioning film; 2, planktonic cell transport to the surface; 3, adhesion of microbial cells; 4, microcolony and EPS formation; 5, growth and detachment.

6.1.3. Medicinal plants as sources of antimicrobials

Medicinal plants and other natural remedies have been the only treatment options available for the management of various infections for eons. With scientific advancement, however, humans have increasingly become dependent on synthetically formulated therapeutic agents for treatment. Despite this development, a substantial number of available drugs are either of natural product origin, or are based on natural scaffolds.[241] The emergence of resistant microbial species is increasingly endangering treatment, with a host of antibiotics already rendered inactive. This, and the long-term lack of antibiotic discovery, has resulted in increased research activities into the identification of alternative remedies and prevention strategies for bacterial infections from diverse sources including medicinal plants.

Subsequently, several phytochemical classes and subclasses, including alkaloids, phenols, flavonoids, tannins and coumarins, have been identified as having potent antimicrobial ability (Table 14).[235] This has been made possible with the aid of a number of methods that have been standardized for evaluating plant extracts and other chemicals to assess their ability to kill or inhibit microbial growth. The methods include disc diffusion, TLC-bioautography, broth microdilution, time-kill tests, ATP bioluminescence, and flow cytometry.[242] In the current study, preliminary antibacterial activity determination of the extracts was done using the agar disk diffusion assay, whilst their minimum inhibitory concentration (MIC) was evaluated with the broth microdilution method.

Although the agar disk diffusion assay has numerous advantages such as simplicity, low cost, ability to test a wide variation of microorganisms, and the ease associated with data interpretation, it produces semi-quantitative results.[242] Furthermore, it is best suited for water-soluble compounds.[243] Consequently, it may not be suitable for hydrophobic compounds and those with high viscosity, as they may have limited diffusion capability. The long incubation periods employed in the assay could also result in the evaporation of volatile components.[242, 243] The assay is also limited in its ability to distinguish between bacteriostatic and bactericidal effects, since bacterial growth inhibition does not necessarily mean death of bacteria. In this method, antibiotics impregnated into paper discs are evaluated for their ability to inhibit microbial growth in inoculated agar plates (Figure 59).

The broth microdilution assay has some advantages over the disk diffusion assay. Although it is also suited for water-soluble compounds, it allows a determination of the MIC of test substances. Moreover, results obtained could reveal whether the agent exhibits a bactericidal or bacteriostatic effect. To facilitate accurate determination of MIC, viewing devices such as spectrophotometers that make use of several colorimetric methods are available.[244] In the current study, the *p*-INT dye was employed to enhance the accuracy of MICs. Viable bacteria convert the dye to an insoluble formazan compound, resulting in a visible violet precipitate.[245] Hence, the presence of bacteria in the wells was determined by their ability to reduce the *p*-INT dye to violet.

Table 14: Antimicrobial activity of phytochemicals (Adapted from Borges *et al*.).[235]

Phytochemical class	**Subclass**	**Example(s)**	**Mechanism of action**	**Reference**
Phenolic and polyphenolic acids	Phenolic acids	• Benzoic acid (*e.g.* gallic acid) • Cinnamic acid (*e.g.* ferulic acid)	• Cell wall and membrane inhibition • Interfere with the metabolism of bacterial cells • Inhibit enzymes and nucleic acid synthesis • Inactivate microbial adhesins	Cowan,[246] Cushnie and Lamb,[247] Puupponen-Pimiä *et al*.,[248] Muthuswamy and Rupasinghe,[249] Perumal Samy and Gopalakrishnakone,[250] Saleem *et al*.[251]
	Flavonoids	• Catechin • Quercetin • robinetin		
	Tannis	• Ellagitannin		
Terpenoids and essential oils	Monoterpenoids	• Thymol	• Increase the membrane fluidity and permeability • Disturb the membrane embedded proteins • Inhibit ion transport processes	Kubo *et al*.,[252] Dixon,[253] Brehm-Stecher and Johnson,[254] Trombetta *et al*.,[255] Horiuchi *et al*.,[256] Daisy *et al*.[257]
	Sesquiterpenoids	• Farnesol • Nerolidol		
	Diterpenoids	• Totarol		
	Sesterterpenoids	• Oleanolic acid		
Alkaloids		• Berberine • Piperine • Stephanine	• Increase membrane/ cell wall permeability	Kim *et al*.,[258] Deng *et al*.[259]
Peptides	Thionins	• Fabatin	• Disrupt cell membranes	Brogden,[260] Barbosa Pelegrini
	Plant defensins	• Pp-Defensin		

Phytochemical class	Subclass	Example(s)	Mechanism of action	Reference
	Lipid transfer proteins	• Ace-AMP1	• Inhibit the nucleic acids and protein synthesis	*et al*.,[261] Seo *et al*.[262]
	Hevein-and knottin-like proteins	• Ac-AMP1 • Mj-AMP1		
	Snakins	• Snakin-1		
Lectins	Legume lectins	• Phytohema-glutinin • Concanavalin A • isolectin I	• Cell wall inhibitors	Bourne *et al*.,[263] Méndez-Vilas[264]
	Chitin-binding lectins	• Wheat germ agglutinin		
	Type 2 ribosome inactivating proteins	• Ricin		
	Jacalin-related lectins	• Jacalin		
	Amaranthus lectins	• Amaranthin		
Polyacetylenes	Falcarinol-type	• C17-acetylene • Diacetylene falcarindiol	• Membrane inhibitors	Kenny *et al*.[265]
Glucosinolate hydrolysis products	Isothiocyanates	• Allylisothio-cyanate • Benzylisothiocy anate • 2-Phenyl-ethylisothio-cyanate	• Bind to sulfhydryl groups of external proteins of cell membranes	Luciano *et al*.,[266] Aires *et al*.[267]
	Nitriles	• Indole-3-acetonitrile		

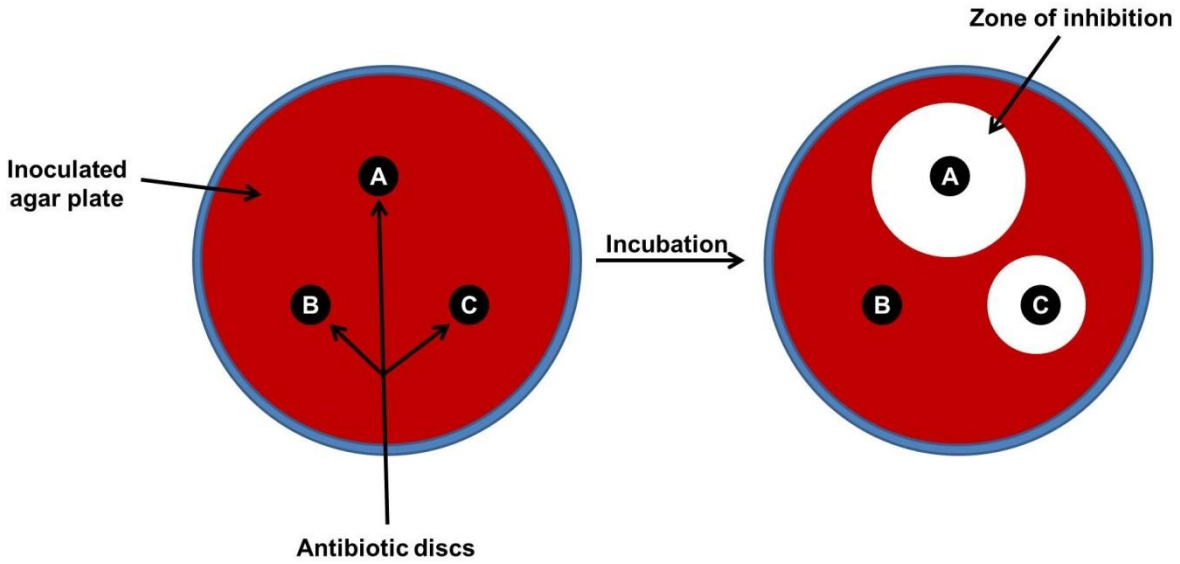

Figure 59: A sketch of the disc diffusion assay showing effect of three antibiotics, A, B, and C on bacterial growth. Antibiotic A is the most active, followed by C. The microorganism is resistant to antibiotic B, or it does not display any noteworthy activity.

6.2. Aim and objectives of chapter

This chapter was aimed at evaluating the antimicrobial effects of extracts prepared from the three ethnomedicinal plants used in wound healing.

The objectives were to

- employ the disc diffusion assay to screen for antimicrobial activity of the extracts against selected bacterial species commonly found in wounds.
- determine the MIC of active extracts using the broth microdilution assay.
- evaluate active extracts for bactericidal and bacteriostatic effects.
- assess the effect of extracts with good antimicrobial activity (MIC ≤ 1 mg/mL) on biofilm biomass.

6.3. Methodology

6.3.1. Microorganisms

All microorganisms were purchased from American Type Culture Collection (ATCC). The bacterial species used were Gram-positive *S. aureus* (ATCC 33591), *E. faecalis* (ATCC 49532), and *S. epidermidis* (ATCC 12228, 35984), as well as Gram-negative

P. aeruginosa (ATCC 19429) and *E. coli* (ATCC 35218). The organisms were maintained on Mueller-Hinton (MH) agar (Merck, SA) at 4°C and cultured in MH broth (Fluka, SA) at 37°C for 24 h prior to commencement of bioassays.

6.3.2. Determination of zone of inhibition

The disc diffusion assay, as described by Bauer *et al*.,[268] was used to determine the inhibitory effect of extracts on each organism. All tests were conducted in accordance with standards set by the Clinical and Laboratory Standards Institute (CLSI, 2009). Plant extract (50 µL, 5 mg/mL) was added to 12.7 mm sterile paper discs (Munktell & Filtrak GmbH, Germany) and the discs dried overnight under a laminar flow cabinet to allow for evaporation of the solvent. Discs impregnated with 5 µg vancomycin (*E. faecalis*) or 5 µg ciprofloxacin (*S. aureus*, *P. aeruginosa*, *E. coli, S. epidermidis)* were included as positive controls (Mast Diagnostics, UK). Vehicle control discs were prepared using 1% DMSO for organic extracts, and sterile distilled water for the water and ethnomedicinal extracts. The inoculum (100 µL, 5×10^5 CFU/mL) was transferred to the surface of Mueller Hinton agar plate and spread evenly across the surface of the petri dish. Zones of inhibition were determined by gently placing the paper discs containing the plant extracts and controls on the inoculated petri dish and incubated for 18 h at 37°C, except for *E. faecalis* which was incubated for 24 h. A digital calliper was used to measure diameter of zones of inhibition, and expressed as the mean diameter of the zone of inhibition (mm) of triplicate measurements.

6.3.3. Minimum inhibitory concentration

The MIC of the extracts was determined using the broth microdilution assay, as previously described.[269] Wells in a 96-well plate were inoculated with 100 µL each of microbial suspension (1×10^6 CFU/mL) and plant extract (0.03 – 1 mg/mL in-reaction) in MH broth. Following 24 h incubation at 37°C, 40 µL of 200 µg/mL aqueous *p*-iodonitrotetrazolium violet (*p*-INT, Sigma-Aldrich, USA) was added to the wells and the cultures incubated for a further 1 h at RT. The MIC was determined as the lowest concentration of extract at which the culture inhibited the conversion of *p*-INT to the purple formazan by reading absorbance at 485 nm (Synergy-2, BioTek).[270]

6.3.4. Minimum bactericidal concentration

The minimum bactericidal concentration (MBC) was determined by subculturing the contents of the first two clear wells obtained in the MIC assay onto agar plates. Plates were incubated at 37°C for 24 h, and the lowest concentration that had no microbial growth was regarded as the MBC.[269]

6.3.5. Determination of effect on biofilms

6.3.5.1. Biofilm culture

Culturing of biofilms was done overnight under sterile conditions as described by Parkar.[271] Fresh 24 h microbial cultures were diluted to 1 x 10^5 CFU/mL with MH broth and 50 µL transferred to all test wells, with the exception of the blank wells to which 50 µL of broth was added and the plate incubated for 24 h at 37ºC.

6.3.5.2. Biofilm inhibition assay

All extracts with an MIC less than or equal to 1 mg/mL were assessed for the ability to inhibit biofilms. To determine the effect of extracts on biofilm growth, each plant extract (0.06 – 1 mg/mL in-reaction) was prepared in MH broth and 50 µL transferred to the test wells of microbial cultures in the micro-plate (from Section 6.5.5.1), and the plate incubated for a further 24 h. Serial dilutions of the antibiotics described in Section 6.3.2 served as the positive controls. After incubation, the content of each well was aspirated, and the wells rinsed three times with distilled water to remove loosely attached cells. The plate was allowed to dry at room temperature, and the crystal violet assay conducted using the method described by Li *et al*. [272] Biofilms were stained for 30 min with 200 µL of 0.1% crystal violet solution at room temperature. Excess stain was rinsed off by washing with distilled water, and the plate air-dried. Stained biofilms were solubilized with 200 µL of 95% ethanol for 5 min, and absorbance read at 560 nm with a spectrophotometer (Synergy-2, BioTek), and biofilm mass estimated as follows:

$$Biofilm\ biomass\ reduction\ (\%) = \left[\frac{Ac - As}{Ac}\right] x100$$

Where *Ac* represents absorbance of negative control, and *As* represents absorbance of test substance.

6.4. Statistical analysis

Data represents results of at least three independent experiments conducted in technical triplicates. Statistical analysis was performed using GraphPad Prism 7 data analysis software.

6.5. Results and discussion

The study evaluated the antibacterial activity of sequential and ethnomedicinal extracts prepared from the three plants under study using the agar disc diffusion and broth microdilution assays. Furthermore, extracts with MIC ≤ 1 mg/mL were tested for effects on bacterial biofilms. Sequential extraction led to a partial fractionation of the antimicrobial compounds in the plants into four solvents with increasing polarity, whilst the ethnomedicinal extract mimicked the traditional preparation. Activity was demonstrated against *E. coli*, *E. faecalis*, and *S. aureus* in the disc diffusion assay. However, *P. aeruginosa* and *S. epidermidis* were resistant to all the extracts tested up to the highest concentration of 250 µg/disc.

6.5.1. *Aspilia africana*

The effect of *A. africana* extracts on growth of bacteria tested using the disc diffusion method is expressed in Table 15. The plant's extracts had activity against two of the six tested bacterial species, the Gram-negative *E. coli*, and the Gram-positive *S. aureus*. Two of the extracts, the hexane and methanol extracts, showed activity against *E. coli* at 250 µg/disc, with recorded zones of inhibition being 15 mm and 16 mm, respectively. Also, the ethyl acetate extract inhibited growth of *S. aureus*, producing a zone of inhibition of 16 mm. However, none of the extracts showed activity against *E. faecalis*, *P. aeruginosa*, and *S. epidermidis*. Assessment of the MICs for the extracts that indicated inhibition, using the broth microdilution assay, indicated that they were all above 1 mg/mL (Table 16). According to previously published criteria, the antibacterial activity of a plant extract is considered to be significant when the MIC is below 100 µg/mL, moderate when the MIC is between 100 µg/mL and 625 µg/mL, or low when MIC is greater than 625 µg/mL.[273] In the current study, the antibacterial

activity exhibited by extracts of *A. africana* could be regarded as low, since their MICs were > 625 µg/mL.

Aqueous and methanol extracts prepared from the leaves of *A. africana* indicated activity against *Aerobacter aerogenes*, *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Clostridium sporogens*, *E. coli*, and *S. aureus*.[274] The *S. aureus*, *B. subtilis*, and *E. coli* bacteria were also susceptible to the petroleum ether, chloroform and methanol crude plant extracts.[275] In an experiment to determine the potential of the plant in wound healing, the authors reported that the methanol leaf extract and the hexane fraction recorded an MIC of 0.5 mg/mL against clinical isolates of *S. aureus*.[74] The study also reported an MIC of 0.125 mg/mL against *P. aeruginosa* for the methanol extract.[74] In a related study, only the aqueous and chloroform extracts showed activity against *S. aureus* and *E. coli.*[276]

Table 15: Zones of inhibition of extracts (mm) against different species of bacteria using the Agar disk diffusion method.

Test substance (250 µg/disc)		**Inhibition of microbial growth (mm)**		
Plant name	**Extract**	***E. coli***	***E. faecalis***	***S. aureus***
A. africana	Hexane	14.67 ± 0.01	-	-
	Ethyl acetate	-	-	15.75 ± 0.05
	Methanol	16.09 ± 0.01	-	-
	Water	-	-	-
	Ethnomedicinal	-	-	-
B. diffusa	Hexane	-	-	19.62 ± 0.03
	Ethyl acetate	-	15.23 ± 0.01	17.90 ± 0.03
	Methanol	15.29 ± 0.05	-	15.55 ± 0.01
	Water	12.37 ± 0.01	-	14.13 ± 0.01
	Ethnomedicinal	11.84 ± 0.02	-	14.63 ± 0.02
E. senegalensis	Hexane	14.58 ± 0.01	-	17.08 ± 0.03
	Ethyl acetate	-	-	-
	Methanol	15.65 ± 0.02	-	14.34 ± 0.01
	Water	-	-	14.54 ± 0.01
	Ethnomedicinal	18.70 ± 0.01	-	-
Ciprofloxacin		27.68 ± 0.01	-	24.36 ± 0.08
Vancomycin		-	16.14 ± 0.19	-

(-) depicts inactivity at the mass tested.

Although these previous accounts did not use the sequential extraction technique employed in the current study, they illustrate the antimicrobial potential of *A. africana*.

Findings of the previous studies are also indicative of the possible presence of antimicrobial compounds in the plant. The current study has demonstrated that the hexane, ethyl acetate, and methanol extracts have weak antibacterial activity (MIC > 1 mg/mL). Furthermore, the aqueous extract (ethnomedicinal extract) tested in this study did not show activity against *E. coli* and *S. aureus*. This was contrary to findings of two previous accounts.[274, 276] The difference in findings could be due to both mechanistic and sampling site variations. The two previous studies used concentrations which were higher than the 5 mg/mL tested in the current study. Whilst one of the studies tested the extracts at 100 mg/mL,[274] the other used 20 mg/mL.[276] These findings suggest that the extracts could have activity at much higher concentrations. However, these concentrations are very high, and not achievable physiologically if ingested. Although such high concentrations could be applied topically, there is still a limit since dermal absorption takes place and may have safety implications.

Various phytochemical groups such as alkaloids, detected in the hexane and ethyl acetate extracts; tannins, in the ethyl acetate and methanol extracts; as well as phenols and flavonoids, detected in the methanol extracts (Table 6), have previously been shown to have antibacterial activities.[235] Furthermore, UPLC-MS evaluation of extracts from the plant confirmed the presence of ascorbic acid and quercetin (Figure 15). Though quercetin, a compound with known antimicrobial activity was detected in the plant (Figure 15),[247, 277] it was not detected in any of the extracts that exhibited antibacterial activity. Hence it is not likely to be responsible for the observed effect. However, the presence of ascorbic acid in the hexane extract of the plant could contribute to the inhibitory effect of the extract against *E. coli*. Although the compound is not known to have direct antibacterial effect, it has the potential to enhance the activity of other antimicrobial compounds present.[278] The compounds directly responsible for the observed effects are therefore not evident from the current findings. Further research activities will be required to unravel the identity of the active compounds.

Table 16: Minimum inhibitory concentrations of extracts (mg/mL) in different bacterial species.

Test substance		Microorganism		
Plant name	**Extract**	***E. coli***	***E. faecalis***	***S. aureus***
A. africana	Hexane	> 1	-	-
	Ethyl acetate	-	-	> 1
	Methanol	> 1	-	-
	Water	-	-	-
	Ethnomedicinal	-	-	-
B. diffusa	Hexane	-	-	> 1
	Ethyl acetate	-	> 1	> 1
	Methanol	> 1	-	> 1
	Water	> 1	-	> 1
	Ethnomedicinal	> 1	-	> 1
E. senegalensis	Hexane	> 1	-	> 1
	Ethyl acetate	-	-	-
	Methanol	0.5	-	1
	Water	-	-	> 1
	Ethnomedicinal	> 1	-	-
Ciprofloxacin		0.00125	-	0.0025
Vancomycin		-	0.0025	-

(-) depicts inactivity at the highest concentration tested.

6.5.2. *Boerhavia diffusa*

The *B. diffusa* extracts were active against three of the five bacterial species tested in the disk diffusion assay (Table 15). The most susceptible microorganism was *S. aureus*, with all five plant extracts inhibiting growth. Whilst *E. faecalis* was only susceptible to the ethyl acetate extract, the methanol, water and ethnomedicinal extracts of the plant were active against *E. coli*. The most significant effect was recorded after treatment with the hexane and ethyl acetate extracts against *S. aureus*, with their zones of inhibition being comparable to ciprofloxacin, the positive control antibiotic used. The inhibition zone diameter recorded for these samples was only 4 mm ($p < 0.05$) and 6 mm ($p > 0.05$) less than the positive control, respectively. Although the inhibition zone obtained for the other treatments was minimal, it is possible that they could exhibit substantial inhibition at higher concentrations such as that used in ethnomedicinal practice. However, the MICs recorded for the active extracts indicated weak activity, being more than 1 mg/mL (Table 16).

A methanol extract of the leaves of *B. diffusa* inhibited *P. aeruginosa*, *B. subtilis* and *S. aureus* at 225 mg/mL in a study using the disc diffusion assay.[279] The extract was also found to have an MIC of 10 mg/mL against *S. aureus*.[279] Whilst this study is in agreement with the current study with respect to activity against *S. aureus*, it also suggests possible effect against *P. aeruginosa* at higher concentrations. Contrary to the current findings, however, no activity was observed against *E. coli* up to the 225 mg/mL used in the previous study.[279] Although the differences in findings between these two studies could be ascribed to the different extraction techniques used, amongst others, it also suggests that fractionation could enhance the antibacterial activity of the extracts. This could particularly be true for activity against *S. aureus*. Whereas the current study indicates the MIC of all extracts to be above 1 mg/mL, the value from the published account was 10 mg/mL. Findings from a related study also suggests that the methanol extract of the aerial parts of the plant inhibited *S. aureus* at 500 µg/disc in the disk diffusion test.[117] This further adds credence to the antibacterial potential of extracts from the plant.

Many phytochemical compounds, including kaempferol, quercetin, and rutin were detected in extracts prepared from the plant (Figures 16 – 17). All three compounds have been previously shown to have activity against some microbial species.[277, 280, 281] Kaempferol and quercetin have both shown good activity against *E. faecalis and P. aeruginosa* (MIC = 50 to 100 µg/mL).[277] Also, moderate (zone of inhibition less than 1 mm) and high (zone of inhibition between 4 and 5 mm) activity were recorded against *E. coli* when treated with 30 µg/disc rutin and quercetin, respectively.[280] In a related study, rutin was reported to show strong activity (MIC = 4 to 16 µg/mL) against *E. coli*, *P. aeruginosa*, and *S. aureus*.[281] Therefore, the effect of the methanol, water and ethnomedicinal extracts against *E. coli* could be attributed to the presence of quercetin and rutin. On the other hand, whilst rutin may account for the activity of the extracts against *S. aureus*, the presence of the other compounds could contribute to the observed effect. Although there appear to be no report on the effect of the detected compounds on *E. faecalis*, the activity observed with treatment of the bacteria with the ethyl acetate extract may be ascribed to the presence of rutin in the extract.

It should also be noted that although the pure compounds identified in the extracts were reported to have exhibited moderate to strong effects on the organisms tested,

the MICs of all the active extracts tested in the current study indicated low activity (MIC > 1 mg/mL) against the bacteria. For instance, the MIC of rutin when treated with *S. aureus* was reported to be 4 µg/mL.[281] However, the MIC recorded for all the extracts with activity against this organism, including ethyl acetate and methanol extracts which indicated presence of rutin, was greater than 1 mg/mL. This could be due to the concomitant presence of other compounds which may be antagonising the activity of active compounds. It also suggests that purification of the extracts could result in enhanced antibacterial potential.

6.5.3. *Erythrina senegalensis*

The effect of *E. senegalensis* extracts on bacterial growth, as assessed by the disk diffusion assay, is indicated on Table 15. The hexane, methanol and ethnomedicinal extracts of the plant inhibited growth of *E. coli*, whilst *S. aureus* was susceptible to the hexane, methanol, and water extracts. The most active extract was the methanol extract, with an MIC of 0.5 mg/mL against *E. coli* (Table 16). The extract also recorded an MIC of 1 mg/mL against *S. aureus*. Some growth was however observed upon overnight re-culturing of clear wells, an indication that the extract was bacteriostatic against *E. coli* and *S. aureus* at the highest tested concentration (1 mg/mL).

The ethanolic extract of roots of *E. senegalensis* was found to inhibit the growth of three Gram-positive bacterial strains; *B. subtilis*, *E. faecalis*, and *S. aureus,* with MICs being between 12 and 23 µg/mL.[282] No effect was observed in the Gram-negative bacteria tested; *E. coli* and *P. aeruginosa,* when treated with the extract up to a concentration of 1500 µg/mL.[282] Whilst these findings suggest that extracts from the roots could be more effective in the management of Gram-positive infections, results of the current experiment indicates that extracts prepared from the leaves could have a broader spectrum of activity. In a related study, the methanol extract from the plant's leaves was found to have an MIC of 125 µg/mL against *S. aureus.*[283] The effect on other micro-organisms tested including *E. coli, Klebsiella pneumoniae*, *P. vulgaris*, *Salmonella typhimurium*, and *S. pyogenes* was not noteworthy.[283] These findings further attest to the antibacterial potential of extracts from the plant. Although differences in solvents, extraction procedure, and plant parts used could be responsible for the disparity in findings between the current and previous studies,

extracts from the present study appear to, additionally, have activity against the Gram-negative *E. coli*. This could be a result of the partial fractionation of the antimicrobial compounds in the plant because of the sequential extraction procedure used in the current study. Purification of crude extracts has been shown to alter activity.[259] Whilst some samples become more active upon purification, others either lose activity or exhibit altered bioactivity.[114, 183, 259]

Kaempferol, neobavaisoflavone, rotenone and rutin, were identified in extracts of the plant using UPLC-MS (Figures 18 and 19). Kaempferol and rutin, which were detected in the methanol extracts of the plant could account for the good activity observed against *E. coli* and *S. aureus*. Both compounds have previously been found to be effective against various microbial species, including *S. aureus*.[277, 281] Although the ethyl acetate extract was also found to contain rutin, none of the tested bacterial strains was susceptible to the extract. Therefore, the presence of other antimicrobial compounds in the extracts cannot be excluded.

6.5.4. Antibiofilm activity

All extracts with MICs less or equal to 1 mg/mL were assessed for possible antibiofilm effects against the susceptible organisms. *E. coli* and *S. aureus* biofilms were therefore cultured and exposed to the methanol extract of *E. senegalensis* at concentrations between 0.06 mg/mL to 1 mg/mL. Treatment with the positive control at a minimum concentration of 0.1 µg/mL led to inhibition of biofilms. This was evident from the clear wells obtained after crystal violet staining (Figure 60). The methanol extract of *E. senegalensis*, however, had no effect on biofilm growth even at the highest concentration tested (1 mg/mL). This could be due to the inability of the extract to penetrate the outer EPS matrix covering of biofilms. No previous reports detailing the effect of extracts from *E. senegalensis* on biofilms could be obtained from literature. However, it is possible that the extract could inhibit biofilms at higher concentrations.

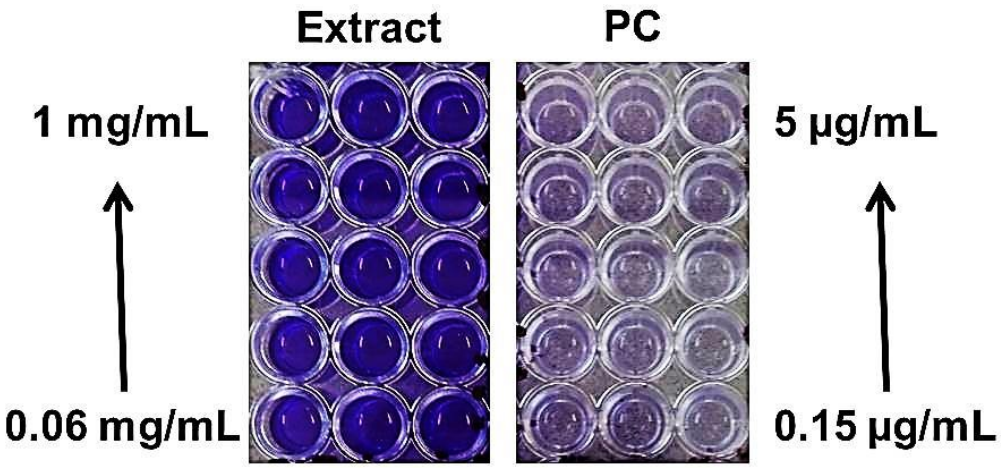

Figure 60: Effect of the methanol extract of *E. senegalensis* on biofilm formation. PC, positive control.

6.6. Conclusion

Most of the extracts inhibited *S. aureus* and *E. coli* growth, whilst only the ethyl acetate extract of *B. diffusa* inhibited growth of *E. faecalis*. The extract with the most promising antibacterial activity was the methanol extract of *E. senegalensis*, with MICs of 0.5 mg/mL and 1 mg/mL against *E. coli* and *S. aureus*, respectively. Although this extract was unable to inhibit biofilms of either organism at 1 mg/mL, it is possible that this could be achieved at higher concentrations. This is, however, subject to verification in subsequent experiments. On the other hand, *P. aeruginosa* and *S. epidermidis* were not susceptible to any of the extracts. Compared to the other plants studied, *E. senegalensis* could have a better antibacterial potential. However, this study has revealed that extracts from the plant could have cytotoxicity implications at concentrations above 100 µg/mL. Therefore, animal experimentation would be required to assess the safety of the extracts at such high concentrations.

Chapter 7

General conclusions, limitations and recommendations

7.1. Conclusions

This study was aimed at evaluating extracts prepared from three Ghanaian plants, anecdotally described as having wound healing potential, on bioactivities such as cellular migration, inflammation, oxidation and bacterial growth, which are important for successful wound healing. Extracts were prepared using two parallel techniques, ethnomedicinally (cold water extraction) and sequentially with four different solvents (hexane, ethyl acetate, methanol, water) in increasing polarity.

Fingerprint analysis using UPLC-MS led to the tentative identification of several compounds for the first time in the plants. These include quercetin in *A. africana*, rutin in *B. diffusa*, as well as kaempferol, rutin, and rotenone in *E. senegalensis*. Some previously identified compounds were also observed in the extracts. Ascorbic acid was detected in *A. africana*, kaempferol and quercetin in *B. diffusa*; and neobavaisoflavone in *E. senegalensis* extracts.

All extracts had an IC_{50} above 100 µg/mL in the three cell types studied, according to the SRB staining assay. Some of the extracts produced a short-term inhibition on growth of cells, whereas others like the ethnomedicinal extracts of *E. senegalensis* demonstrated potential to be cytotoxic at concentrations more than 100 µg/mL. Microscopic evaluation of extract-treated cells also indicated no cytotoxic effects.

Generally, the results suggest a better antioxidant activity with the more polar extracts. The most prominent effect was observed with the ethyl acetate extract of *B. diffusa*, whilst the hexane extracts of all the plants yielded the poorest outcome. The ethyl acetate extract of *B. diffusa*, ethnomedicinal extract of *E. senegalensis*, and the ethyl acetate extracts of *A. africana*, recorded the most prominent effect against AAPH-induced oxidation in myoblasts, fibroblasts, and macrophages, respectively. Furthermore, none of the extracts had a prominent inhibitory effect on XO activity.

Cellular migration was altered by most of the extracts, though marginally in some instances. Enhancement of cellular migration could, therefore, be one of the wound healing mechanisms of the plants under study.

Analysis using the disc diffusion assay indicated that a lot of the extracts had ability to inhibit *E. coli* and *S. aureus*, albeit at higher concentrations (250 µg/disc). The most prominent effect was exhibited by the methanol extract of *E. senegalensis* against *E. coli* (MIC = 0.5 mg/mL). However, no effect on biofilm growth was observed upon treatment with this extract up to a maximum concentration of 1 mg/mL. Therefore, although the extracts could be useful in treating infected wounds, this can only be achieved at concentrations above 1 mg/mL.

Hence, extracts prepared from the three plants under study have demonstrated activity against free radicals, oxidative stress, inflammation, microbial organisms, as well as ability to facilitate cellular migration. Although most extracts exhibited antioxidant activity, the ethyl acetate extract of *B. diffusa* was most prominent. This extract also reduced AAPH-induced oxidative stress in a concentration-dependent manner, suggesting that it could be useful in the management of ROS-induced wounds. However, the extract could affect myoblast activity by inducing a temporal block in growth of the cells at high concentrations, and therefore must be administered cautiously. Also, the ethyl acetate extract of *E. senegalensis* was the best at inhibiting xanthine oxidase activity, indicating anti-inflammatory potential. Whilst this extract may be useful in suppressing inflammation, it could also suppress growth of other important cells such as fibroblasts, in addition to possibly pre-disposing the wound to infection. The hexane extract of *A. africana* could enhance wound healing by facilitating activity of myoblasts, and protection against bacterial infection. Furthermore, the methanol extract of *E. senegalensis* could also protect against bacterial infection, although its use at such high concentrations could result in cytotoxicity. Therefore, although the findings of this study contribute some scientific data to support the ethnomedicinal use of the three plants as wound remedies, practitioners should be advised against the use of high concentrations because of safety implications.

7.2. Study limitations and recommendations

Cytotoxicity assessment of extracts in the study was conducted over a maximum period of 48 h. Although this enabled short-term cytotoxic manifestation of extracts, their long-term effects on the cells could only be inferred. Extracts should be tested for a longer period to ascertain the extent of cytotoxicity.

Intracellular ROS was measured using DCF-DA as a fluorescent marker. As this is only one method of assessing cellular ROS levels, other methods such as flow cytometry should be explored for comparative purposes. By comparing results from two or more methods, a broader picture on the effect of the extracts would be obtained. Through these analysis, the extent of effect, and mechanisms involved could be obtained.

Also, only one inflammatory assay, XO inhibitory activity, was conducted. Since chemical entities could exhibit anti-inflammatory activity through various mechanisms, extracts should be explored for their ability to inhibit other inflammatory processes such as cyclooxygenase and cytokine release.

Effect on wound closure was assessed *in vitro* using the scratch assay. This assay provides useful preliminary information on the possible wound healing effect of chemical substances. However, it is limited to the extent that the wound is devoid of all the complexities of an actual wound. Hence the findings may not necessarily be reproduced in actual wounds. Further work should be conducted using wounds induced in laboratory animals, as they are more related physiologically to humans.

The antibacterial and antibiofilm experiments conducted in this study were aimed at extracts with moderate to high activity against the microbial organisms. This led to the use of concentrations up to a maximum of 1 mg/mL in the microdilution and anti-biofilm assays. Although this approach is generally acceptable, it could lead to elimination of extracts with weak activity. To forestall this, future experiments should be conducted using higher concentrations. This will enable the identification of all active extracts, the purification of which could lead to the discovery of more active compounds. Also, this

study tested the effect of the extracts in only six micro-organisms. This should be expanded to cover other organisms such as fungi and yeasts.

Lastly, although the study identified some phytochemical compounds in the extracts, their presence could not be confirmed using other techniques. This should be carried out in future experiments. Also, since the compounds were not directly tested in this study, their activity could only be inferred. Hence, future studies should be directed towards the bio-assay guided isolation of the identified, as well as other novel, compounds that may be present. Such compounds should be tested directly to confirm their role in the plants activity.

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Appendix I: Publications

South African Journal of Botany 118 (2018) 11–17

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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 phyto-chemical 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 fibro-blasts, 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 demon-strated 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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Research Paper

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^{0}-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 lg/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 lg/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 protec-tion 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 com-plex 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 myo-blast alignment and their fusion into myotubes. This pro-cess 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

Comparative Effect of Three Wound Healing Ethnomedicinal Extracts on Fibroblast Migration and Reactive Oxygen Species Release *In vitro*

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Background: A large proportion of the world's human population relies on medicinal plants for primary health care. Three of the plants commonly used for managing wounds in Ghana and other tropical countries are *Aspilia africana* (AA), *Boerhavia diffusa* (BD), and *Erythrina senegalensis* (ES). This study was aimed at studying the effect of the plants on fibroblast migration and intracellular reactive oxygen species (ROS) release. **Methods:** Water extracts, simulating ethnomedicinal preparations were screened for cytotoxicity in SC-1 fibroblasts using the sulforhodamine B assay and microscopy (live/dead staining, Phase contrast, and PlasDIC). Oxidative stress was induced with 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), and ROS release measured every minute for 2 h using 2,7-dichlorofluorescein diacetate. Wounds were generated in a mono-layer cell culture and migration assessed at 0, 8 and 24 h using microscopy. **Results:** All extracts exhibited negligible cytotoxicity. AAPH increased ROS release by 72.3% relative to the negative control. Pretreatment with 100µg/ mL of AA, BD, and ES resulted in up to 67.1%, 28.8%, and 69.0% reduction in ROS release, respectively. Treatment with 100 µg/mL of BD and ES reduced fibroblast migration from 64.4% in the untreated cells to 50.4% and 43.5%, respectively, while AA had no observable effect. **Conclusion:** The ethnomedicinal extracts have all demonstrated potential to suppress ROS release, an effect which could facilitate healing of wounds with oxidative stress occurring. All extracts, except AA, negatively affected fibroblast migration which could delay wound healing. Beneficial effects on wound treatment may thus be related to antioxidant activity, and not cellular migration.

End of Day 2

Appendix II: Ethical approval

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

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YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

1/06/2017

Approval Certificate
New Application

Ethics Reference No.: 194/2017

Title: The effect of three Ghanaian plants on fibroblast migration, inflammation and bacterial growth In Vitro

Dear Mr Ewura Seidu Yahaya

The **New Application** as supported by documents specified in your cover letter dated 3/05/2017 for your research received on the 3/05/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 31/05/2017.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years
- Please remember to use your protocol number (**194/2017**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers, MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

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Appendix III: Preparation of reagents

AAPH solution

AAPH was purchased from Sigma-Aldrich (Saint Louis, USA). A 1 mM stock solution was prepared by dissolving 0.027 mg AAPH powder in 100 µL PBS. Aliquots of the stock solution were stored at 4ºC and used the same day.

ABTS free radical solution

The ABTS free radical solution was prepared by mixing 7 mM ABTS and 2.4 mM potassium persulfate, both purchased from Sigma-Aldrich (Saint Louis, USA). The solution consisted of 18.01 mg ABTS and 3.24 mg potassium persulfate in 5 mL distilled water, and was stored in the dark at 4ºC.

Allopurinol

Allopurinol was purchased from Sigma-Aldrich (Saint Louis, USA). A 1 mM stock solution was prepared by dissolving 0.136 mg in 50 µL DMSO and diluting to 1 mL with phosphate buffer. The solution was kept at -20ºC until used.

Aluminium trichloride reagent

Aluminium trichloride was purchased from Merck Chemicals (South Africa). A 1% ethanolic solution was prepared by adding 100 mg aluminium trichloride to 10 mL absolute ethanol.

Anisaldehyde-sulphuric acid reagent

Anisaldehyde solution was purchased from Sigma-Aldrich (Saint Louis, USA). The reagent was prepared just before spraying by mixing anisaldehyde solution with acetic acid, methanol, and concentrated sulphuric acid in a ratio of 0.1:2:17:1.

Dichlorofluorescein diacetate

Dichlorofluorescein diacetate was purchased from Sigma-Aldrich (Saint Louis, USA). A stock solution of 10 mM was prepared by dissolving 4.85 mg in 1 mL DMSO. Aliquots of the stock solution were stored at -20ºC.

Dulbecco's Modified Eagle's Medium

Powdered DMEM was purchased from Sigma-Aldrich (Saint Louis, USA). A 1.3% solution was prepared by dissolving 67.35 g DMEM powder and 18.5 g sodium bicarbonate (Merck, Germany) in 5 L sterile, deionised water. The solution was filter-sterilised twice (Sartorius, 0.22 µm), supplemented with 1% penicillin/streptomycin (BioWhittaker, Walkersville, USA), and stored at 4ºC until used.

DPPH free radical solution

DPPH was purchased from Sigma-Aldrich (Saint Louis, USA). A 0.135 mM solution was prepared by dissolving 2.66 mg DPPH in 50 mL distilled water prior to use.

Dragendorff's reagent

Bismuth nitrate and potassium iodide were purchased from Merck Chemicals, South Africa. The reagent was made up of two solutions. Solution A was prepared by dissolving 0.85 g bismuth nitrate in 50 mL of 25% aqueous acetic acid. Solution B was prepared by dissolving 8 g potassium iodide in 30 mL water. Fresh working solution of Dragendorff's reagent was prepared before spraying by adding solution A and B in a ratio of 5:3.

Fluorescein diacetate

Fluorescein diacetate was purchased from Sigma-Aldrich (Saint Louis, USA). A 5 mg/mL stock solution was prepared in acetone and stored at -20ºC. A 2 µg/mL working solution was prepared by diluting 2 µL of the stock solution in 5 mL PBS.

Foetal calf serum

FCS was acquired from The Scientific Group (Gauteng, South Africa), and heat inactivated at at 56ºC for 45 min. Solutions were stored at -20ºC until used.

Folin-Ciocalteu reagent

A solution of Folin-Ciocalteu was purchased from Sigma-Aldrich (Saint Louis, USA). The working reagent consisted of 10% (v/v) Folin-Ciocalteu in distilled water.

Iron-III-chloride reagent

Iron-III-chloride was purchased from Merck Chemicals (South Africa). The reagent was prepared by dissolving 0.5 g iron-III-chloride in 2.5 mL water, and diluting to 50 mL with ethanol.

Mueller-Hinton agar

Mueller-Hinton agar was purchased from Davies Diagnostics (Randburg, South Africa). The agar was prepared by dissolving 18 mg powder in 500 mL distilled water and autoclaved at 121 ºC for 15 min. Agar was used after cooling to 50 – 55ºC.

Mueller-Hinton broth

Mueller-Hinton broth was purchased from Davies Diagnostics (Randburg, South Africa). The broth was prepared by dissolving 12 mg powder in 500 mL distilled water and autoclaved at 121ºC for 15 min. The broth was stored at 4ºC until use.

Phorbol 12-myristate-13- acetate

A 1 mg film of phorbol-12-myristate 13-acetate was purchased from Sigma-Aldrich (South Africa). The entire content was dissolved in 1 mL DMSO and aliquots stored at -20ºC until used.

Phosphate buffer

Phosphate buffer (pH = 7.5) was prepared from potassium phosphate dibasic (K_2HPO_4) and potassium phosphate monobasic (KH_2PO_4), both purchased from Merck chemicals (South Africa). A 1 M stock solution was prepared from both salts in separate containers by dissolving 17.42 g and 13.61 g, respectively, in 100 mL distilled water. The 0.1 M phosphate buffer solution was prepared by mixing 86.6 mL K_2HPO_4 and 13.4 mL KH_2PO_4 in a volumetric flask, and diluting to 1 L with distilled water.

Phosphate buffered saline

BBL™ FTA haemagglutination buffer was obtained in powdered form from BD Scientific (Paris, France). A 0.9% (w/v) solution was prepared by dissolving 4.5 g powder in 500 mL distilled water. The solution was stored at 4ºC.

Phytochemical standards

Ascorbic acid, kaempferol, limonene, quercetin, neobavaisoflavone, and rutin were purchased from Sigma-Aldrich (Saint Louis, USA). A working concentration of 1 mg/mL solution was prepared by dissolving 1 mg powder in 1 mL methanol.

***p*-Iodonitro tetrazolium**

p-INT was purchased from Sigma-Aldrich (Saint Louis, USA). A working solution of 200 µg/mL was prepared prior to use by dissolving 0.1 g of powdered *p*-INT in 500 mL distilled water.

Potassium hydroxide reagent

Potassium hydroxide was purchased from Merck Chemicals (South Africa). The reagent consisted of 5% ethanolic solution of 500 mg/mL aqueous potassium hydroxide. This was done by dissolving 5 g of potassium hydroxide in 10 mL 5% ethanolic solution.

Propidium iodide

Propidium iodide was purchased from Sigma-Aldrich (Saint Louis, USA). A stock solution of 2 mg/mL was prepared in PBS and stored at at 4ºC, from which a 10 µg/mL working solution was prepared by diluting 25 µL of the stock to 5 mL with PBS.

Roswell Park Memorial Institute-1640 medium

RPMI solution was purchased from Sigma-Aldrich (Saint Louis, USA). The solution was supplemented with 2 mM L-glutamine (Sigma-Aldrich, USA), 10 mM HEPES (Sigma-Aldrich, USA), 1 mM sodium pyruvate (Sigma-Aldrich, USA), 2.5 g/L glucose (Merck chemicals, South Africa), 50 µM 2-mercaptoethanol (Sigma-Aldrich, USA), and 1% penicillin/streptomycin (BioWhittaker, Walkersville, USA).

Solvents

Benzene, butanone, chloroform, dimethyl sulfoxide, ethanol, ethyl acetate, hexane, and methanol were purchased from Merck (Pty) Ltd, South Africa, stored at room temperature, and used undiluted. LC-MS grade acetonitrile and formic acid were purchased from Sigma-Aldrich (Saint Louis, USA). Acetic acid, hydrochloric acid, and sulphuric acid were purchased from Merck Chemicals (South Africa).

Sulforhodamine B

Sulforhodamine B was acquired from Sigma Aldrich (Saint Louis, USA). A 0.057% (w/v) solution was prepared by dissolving 0.285g powder in 1% acetic acid to a volume of 500 mL, and stored at 4ºC until used.

Trichloroacetic acid

Trichloroacetic acid powder was obtained from Merck Chemicals (South Africa). A 50% (w/v) solution was prepared by dissolving 50 g samples in distilled water to a total final volume of 100 mL, and stored at 4ºC until used.

Trypan blue

Trypan blue was purchased from Sigma-Aldrich (Saint Louis, USA). A 0.1% (w/v) solution was prepared by dissolving 0.01 g in distilled water to a final volume of 10mL, filtered through 0.4 µm pore size filter, and stored at room temperature.

Trypsin/Versene

Trypsin/Versene was purchased from The Scientific Group (Gauteng, South Africa), and used undiluted. The solution was stored at 4ºC.

Xanthine

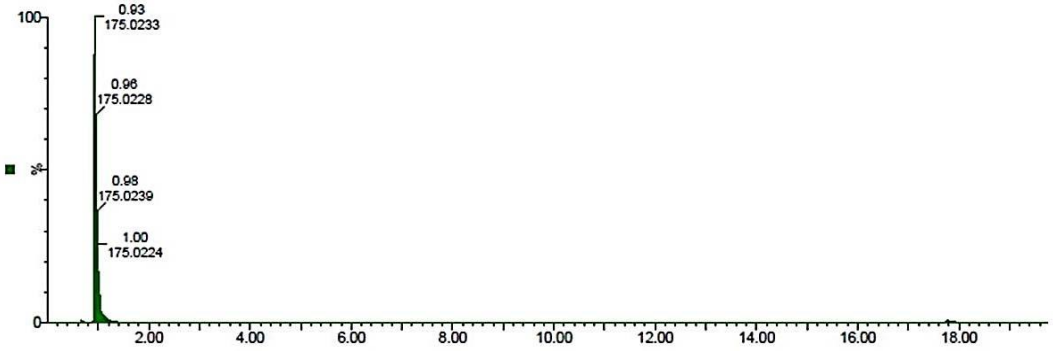
Xanthine was purchased from Sigma-Aldrich (Saint Louis, USA). A 32.87 mM stock solution was prepared in 1 M sodium hydroxide, from which 0.3 mM working solution was prepared by diluting 91.2 µL to 10 mL with phosphate buffer when required.

Xanthine oxidase

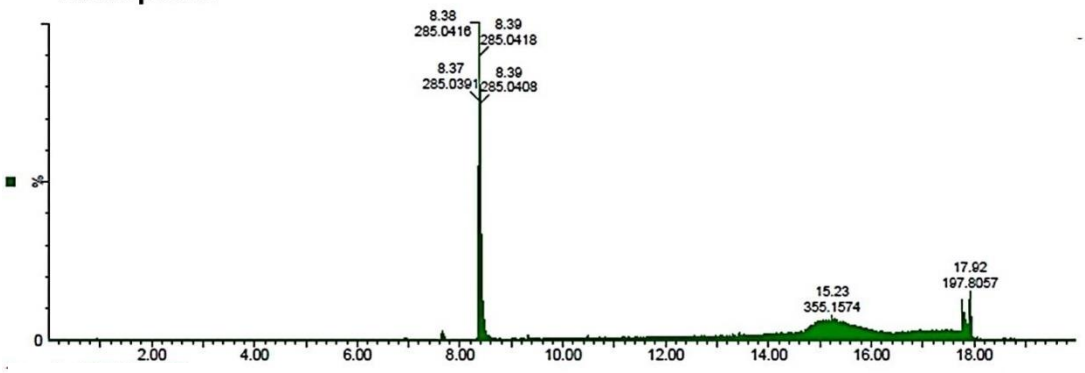
Xanthine oxidase was purchased from Sigma-Aldrich (Saint Louis, USA). A working solution of 0.02 units/mL was prepared from a 4.62 units/mL stock solution by diluting 4 µL enzyme solution to 1 mL with phosphate buffer and stored on ice.

Appendix IV: UPLC-MS chromatograms of phytochemical standards

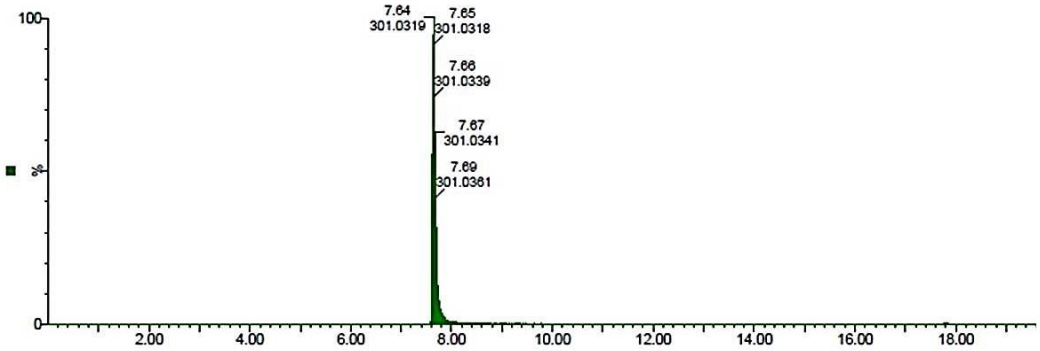
Ascorbic acid



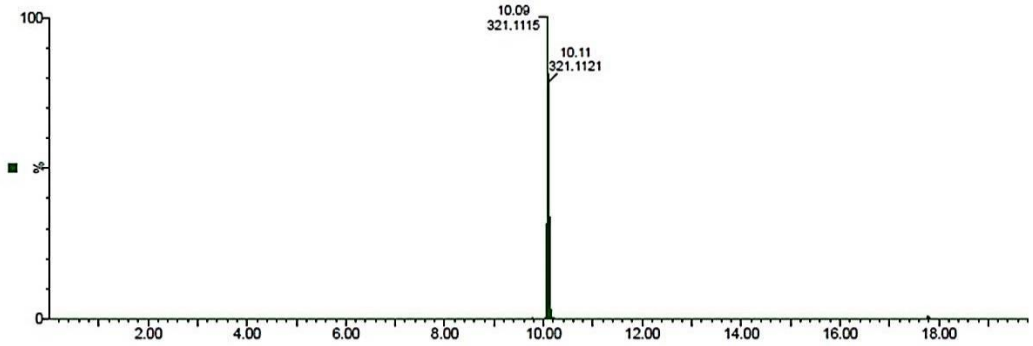
Kaempferol



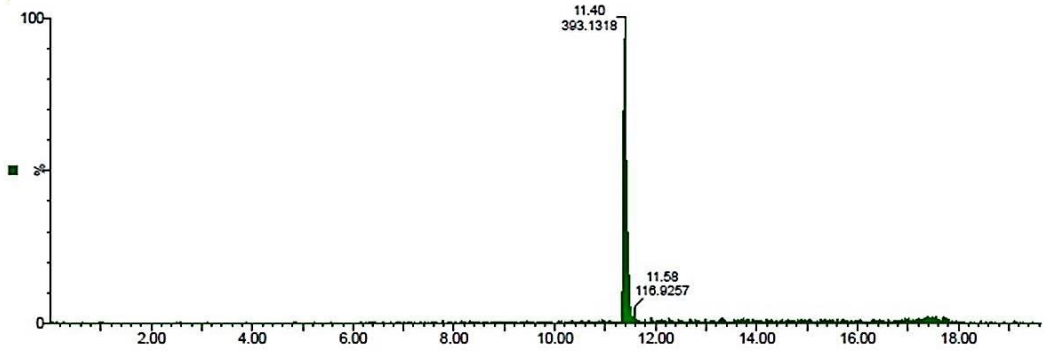
Quercetin



Neobavaisoflavone



Rotenone



Rutin