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Wound Healing Potential of *Terminalia sericea*

by

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Declaration

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Dedication

To Hajiera Parkar, who was taken from us too soon…

For teaching me the importance of independence through education and making me the women I am today.

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All that I have accomplished thus far and all that I am still trying to achieve, would not be possible without the blessings and the guidance of the Almighty Allah (SWT).

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"The women who follows the crowd will usually go no further than the crowd. The women who walks alone is likely to find herself in places no one has ever been before"

~ Albert Einstein

Table of Contents

Abstract

A wound occurs when the integrity of the tissue is compromised, resulting in the breakdown of its protective functions. Wound healing is a complex sequential overlapping process. The progression of the wound healing process is hindered by bacterial infections and free radicals. Treatments for wounds are available but are accompanied by an increasing resistance. It is for this reason that new sources of treatment are constantly being sought. Plants have been used ethnomedicinally to treat wounds and one such plant is *Terminalia sericea* Burch. ex DC. (Combretaceae). The aim of this study was to assess the wound healing activity of *T. sericea*.

Hot water (HW), methanol (MeOH), ethyl acetate (EtOAc) and hexane (HE) extracts were prepared. Thin layer chromatography (TLC) and ultra-performance liquid chromatography time of flight mass spectrometry (UPLC-TOF-MS) were used to determine the phytochemical classes and genus specific compounds present in the plant. Antioxidant activity was assessed using the $ABTS^{\bullet+}$ and DPPH radical scavenging assays as well as the cellular antioxidant assay. Antibacterial activity was determined using the broth microdilution assay and the biofilm inhibition assay. The extracts were assessed for cytotoxicity after 24 h and 72 h in the SC-1 (fibroblasts) and EA.hy926 (endothelial) cell lines using the sulphorhodamine B (SRB) assay. The ability of the extracts to enhance proliferation and migration in the SC-1 and EA.hy926 cell lines was assessed using the fibroblast proliferation and scratch assays.

The major phytochemical classes detected in the extracts using TLC were alkaloids, coumarins, flavonoids, glycosides, phenolics, saponins, sterols and terpenoids. The genus specific compounds punicalagin, sericoside, anolignan B and arjunic acid were identified in the extracts by means of UPLC-TOF-MS. The MeOH and EtOAc extracts as well as Trolox displayed radical scavenging ability with IC_{50} values of 0.525, 0.387 and 0.694 µg/mL, and 9.080, 12.660 and 13.800 µg/mL against $ABTS^{\bullet+}$ and DPPH, respectively. All extracts exhibited a protective dose dependent decrease in intracellular ROS in response to oxidative damage by AAPH.

A noteworthy MIC of 1 mg/mL against *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus* was noted for the HE extracts. The MeOH,

HW and EtOAc extracts displayed antimicrobial activity at concentrations > 3.13 mg/mL. Most extracts were found to significantly enhance biofilm growth.

A low cytotoxic trend was observed overall. None of the extracts displayed cytotoxic effects after a 24 h exposure period, however after 72 h the MeOH and EtOAc extracts exhibited a prominent reduction in cell density at the higher concentrations. The IC_{50}'s (72 h) for the MeOH and EtOAc extracts were 33.77 and 53.04 µg/mL in the SC-1 cell line and 51.82 and 57.83 µg/mL in the EA.hy926 cell line, respectively. A significant ($p < 0.05$) enhancement of cell migration in both the fibroblast and endothelial hybrid cell lines were displayed in the scratch assay. However, no significant increase in cell density was noted with the fibroblast proliferation assay, with the exception of the EtOAc extract at 1 µg/mL ($p \leq 0.05$). The results from the *in vitro* wound healing assays indicated that the extracts of *T. sericea* enhanced migration but not proliferation.

The overall wound healing effect of *T. sericea* has been attributed to a combination of the effects that it elicits on the multiple processes involved in the wound healing process which include antimicrobial, antioxidant, migratory and proliferative activity. Based on these results, the ethnomedicinal usage of *T. sericea* in the treatment of wounds is validated. Further studies involving the isolation and testing of the active compounds contained within EtOAc and HE extracts are warranted.

Keywords: antimicrobial; antioxidant; cytotoxicity, *Terminalia sericea*; wound healing, migration, proliferation

List of Figures

List of Tables

List of abbreviations

%	Percentage
µg	Microgram
µg/mL	Micrograms per milliliter
µL	Microliter
µM	Micromolar
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
AUC	Area under the curve
BSA	Bovine serum albumin
CAA	Cellular antioxidant activity
Cell/mL	Cells per milliliter
CFU	Colony forming unit
CFU/mL	Colony forming units per milliliter
CLSI	Clinical and Laboratory Standards Institute
cm	Centimeter
CO_2	Carbon dioxide
DCF	2’,7’ –Dichlorofluorescin
DCFDA	2’,7’ –Dichlorofluorescin diacetate
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2.2-Diphenyl-1-1picrylhydrazyl
EGF	Endothelial growth factor
Em	Emission wavelength
ESI	Electrospray ionisation
EtOAc	Ethyl acetate

Ex	Excitation wavelength
FCS	Foetal calf serum
FGF	Fibroblast growth factor
g	Relative centrifugal force
h	Hour/s
H_2-DCFDA	2', 7'-Dichlorodihydrofluorescein diacetate
Half-log	Half-logarithmic
HAT	Hydrogen electron transfer
HE	Hexane
HIV	Human immunodeficiency virus
HPTLC	High performance thin layer chromatography
HW	Hot water
IC_{50}	Half maximal concentration
IL-1β	Interleukin one beta
MALDI	Matrix-assisted laser desorption/ionization
MAPK	Mitogen activated protein kinase
MBC	Minimum bactericidal concentration
mDa	Megadalton
MeOH	Methanol
mg/kg	Milligram per kilogram
mg/kg/day	Milligram per kilogram per day
mg/mL	Milligrams per milliliter
MIC	Minimum inhibitory concentration
min	Minute/s
mL	Milliliter
mm	Millimeter
mM	Millimolar
MS	Mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nm	Nanometer

NRF-2	Nuclear factor erythroid 2-related factor
ºC	Degrees Celsius
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
pg/mL	Picogram per mL
p-INT	*p*- Iodonitrotetrazolium
Rf	Retention factor
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
s	Second/s
SD	Standard deviation
SEM	Standard error of the mean
SET	Single electron transfer
SRB	Sulphorhodamine B
TGF	Transforming growth factor
TGF-β	Transforming growth factor beta
TLC	Thin layer chromatography
TNF-α	Tumour necrosis factor alpha
TOF	Time of flight
Tris	Trisaminomethane
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
w/v	Weight per volume
w/w	Weight per weight

Chapter 1: Literature review

1.1. Overview

The healing of wounds is one of the most expensive medical complications following surgery, and are an ideal environment for bacterial growth which leads to increased rates of nosocomial infections in hospitals.[1] Chronic wounds have a negative impact on patient quality of life which is estimated to be comparable with that of other chronic diseases.[2] Wound healing is a complex process that can be hindered by many factors such as oxidative damage, microbial infection and delayed angiogenesis.[3] The fact that all the biological mechanisms surrounding wound healing are not fully understood, is evident in the lack of a true gold standard for treatment. The emergence of antimicrobial resistance has introduced new challenges in the area of wound care.[4] It is for these reasons that new compounds are continually being sought to aid in the progression of the wound healing process. Plants are a rich and diverse source of bioactive compounds which, if tested properly, could be developed into treatments for a plethora of diseases. Due to the economic and social impact of wounds, a higher level of attention and resources need to be dedicated to the understanding of the biological mechanisms underlying wound complications and the treatment thereof.[1]

1.2. Structure of the skin

The skin (which is the largest organ system in the human body), forms part of the integumentary system, together with hair, nails and the mucosal surfaces of the gastrointestinal tract.[5] The skin primarily functions as a barrier against the environment[6] and serves as the first line of defence against pathogens that can invade the body after its integrity has been compromised by burns, surgical wounds and trauma (such as accidental wounds).[7] It consists of three main layers: the epidermis, dermis and the hypodermis or subcutantaneous layer (Figure 1).[8]

I. The *epidermis*, which is the outer most layer of the skin, functions mainly in protecting the skin from environmental threats (UV light and passive water loss), providing barriers against physical, chemical and microbial damage and serving as innate and adaptive immunologic barriers.[9,10] The epidermis is sub-divided into five

layers: the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and the stratum basale.[5,8]

The predominant cell type in the epidermis is the keratinocyte, which is present in the basal layer where they are constantly being incorporated into the granular layer in which they transform into the flat, anucleated, squamous cells (corneocytes) of the stratum corneum.[9,10] Keratinocytes produce and express various proteins, such as keratin and fillagrin which are the major structural proteins (80-90%) contained in the epidermis. These cells also produce lipids and cytokines which are involved in wound healing.[10,11] Other cell types in the epidermis include melanocytes (found in the basal layer and protect against UV damage), Langerhans cells (present in the stratum spinosum and play an important role in the immune barrier of the skin and contact allergic reactions), as well as lymphocytes (present under certain circumstances such during in an immune response).[10,11]

II. The *dermis* can be divided into the upper papillary and lower reticular dermis.[8] It is a connective layer consisting primarily of collagen and elastin which are produced by fibroblasts housed within the layer.[11] The function of the dermis is to provide mechanical protection and to house specialized structures such as lymph vessels, sweat and sebaceous glands and hair follicles.[10]

III. The *hypodermis* consists of the panniculus adiposus (the fatty layer) and the panniculus carnosis (the vestigial layer of muscle).[5,8] The function of the hypodermis is to protect against mechanical shock, to provide insulation and for energy metabolism and storage.[10]

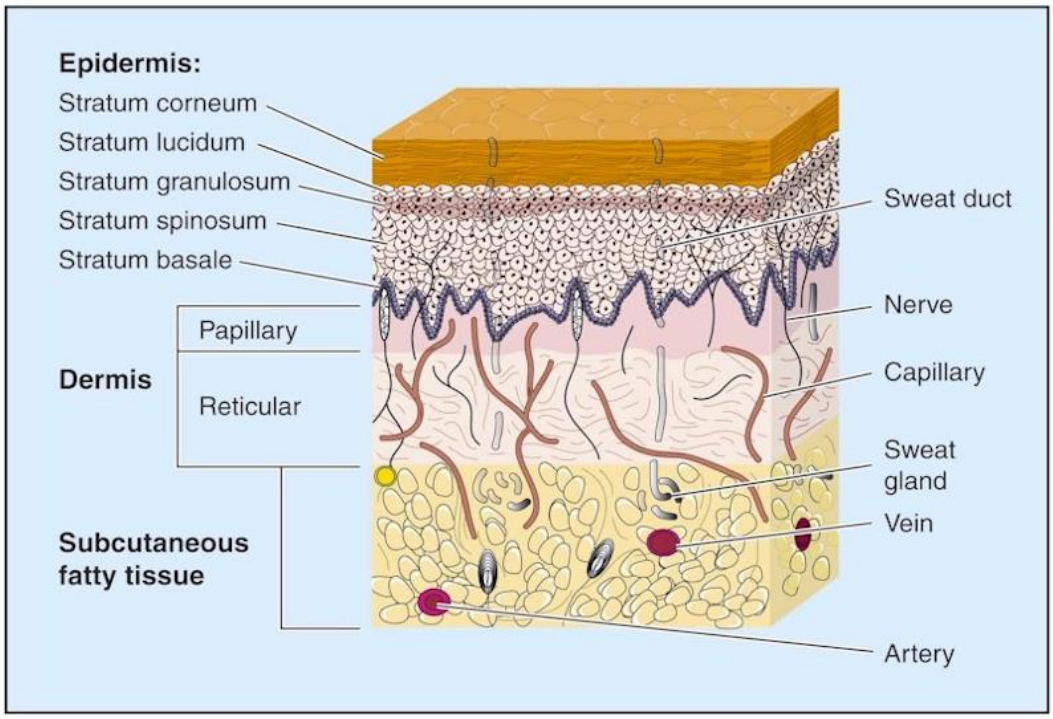


Figure 1*:* Layers of the skin.[12]

1.3. Classification of a wound

A wound occurs when the integrity of the tissue is compromised through surgical incision, laceration, abrasions, avulsions, punctures, bites, contusions or haematomas.[13] It can be defined as a breakdown in the protective function of the skin, the loss of continuity of the epithelium, with or without loss of underlying connective tissue following injury to the skin, underlying tissues or organs.[14] The latter can be caused by surgery, a blow, a cut, a burn (chemicals, heat or cold), friction or shear force, pressure or as a result of disease (leg ulcers or carcinomas).[15] Wounds can also be classified as open, where the skin has been breached exposing the underlying tissue (surgical incisions, lacerations, abrasions, avulsions, punctures and bites) or closed, where the skin remains intact but underlying structures have suffered trauma (contusions and haematomas).[13]

Wounds may be chronic or acute.[13] Acute wounds are tissue injuries that proceed though the wound healing process in an orderly manner within the projected time frame, resulting in proper restoration of the anatomical and functional integrity of the wounded area.[16] Chronic wounds are those injuries which do not progress through the

wound healing process in the expected manner and do not heal within the normal time frame. As a result, these wounds enter a state of pathological inflammation and either keep recurring or require an extensive amount of time and treatment to heal.[16] The most common causes of chronic wounds are local infection, hypoxia, trauma, foreign bodies, diabetes mellitus, malnutrition, immunodeficiency or medications.[16] Another type of wound includes antimicrobial wounds which result from the direct necrotic effect causing tissue damage or the commandeering of the cell function.[7]

1.4. The wound healing process

Wound healing is a complex sequential but overlapping process characterised by four phases: haemostasis, inflammation, proliferation and remodelling (Figure 2).

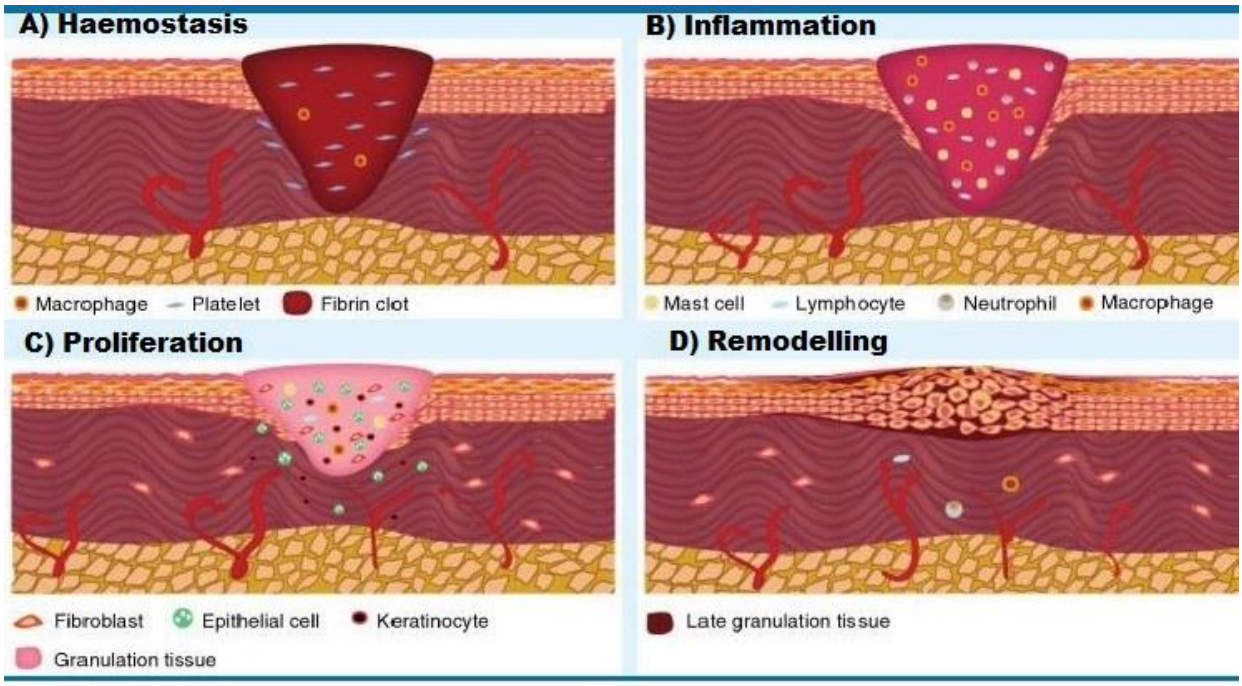


Figure 2: The wound healing process A) haemostasis, B) inflammation, C) proliferation and D) remodelling.[6]

1.4.1. The haemostasis phase

The haemostasis phase (Figure 2) begins when damaged blood vessels bleed into the wound.[17] The damaged blood vessels and adjacent small arteries and arterioles will constrict in an attempt to limit the amount of blood loss.[8,18] Once the bleeding has slowed, histamine released by damaged cells and mast cells cause blood vessels to dilate.[8] This increases blood flow to the area making the blood capillaries more

permeable, which in turn causes blood plasma containing antibodies, clotting proteins, platelets and other blood cells to seep into the wound.[8,13]

The slowed bleeding results in contact activation of blood platelets and coagulation factors.[18] The main function of blood platelets is the formation of mechanical plugs during the normal homeostatic response to vascular injury.[18] Adenosine diphosphate (ADP) causes blood platelets to adhere to exposed collagen I on the sub-endothelial matrix where they become activated, swell and secrete adhesive glycoproteins which leads to platelet aggregation and the formation of a primary haemostatic plug.[16,18] Platelets also secrete factors which stimulate the production of thrombin which initiates the coagulation cascade (Figure 3).[16,18,19] Thrombin converts soluble plasma fibrinogen into fibrin [16,18]. Fibrin forms a mesh which strengthens the platelet aggregate of the primary haemostatic plug into a stable haemostatic plug.[18] This phase lasts 30-45 min.[17]

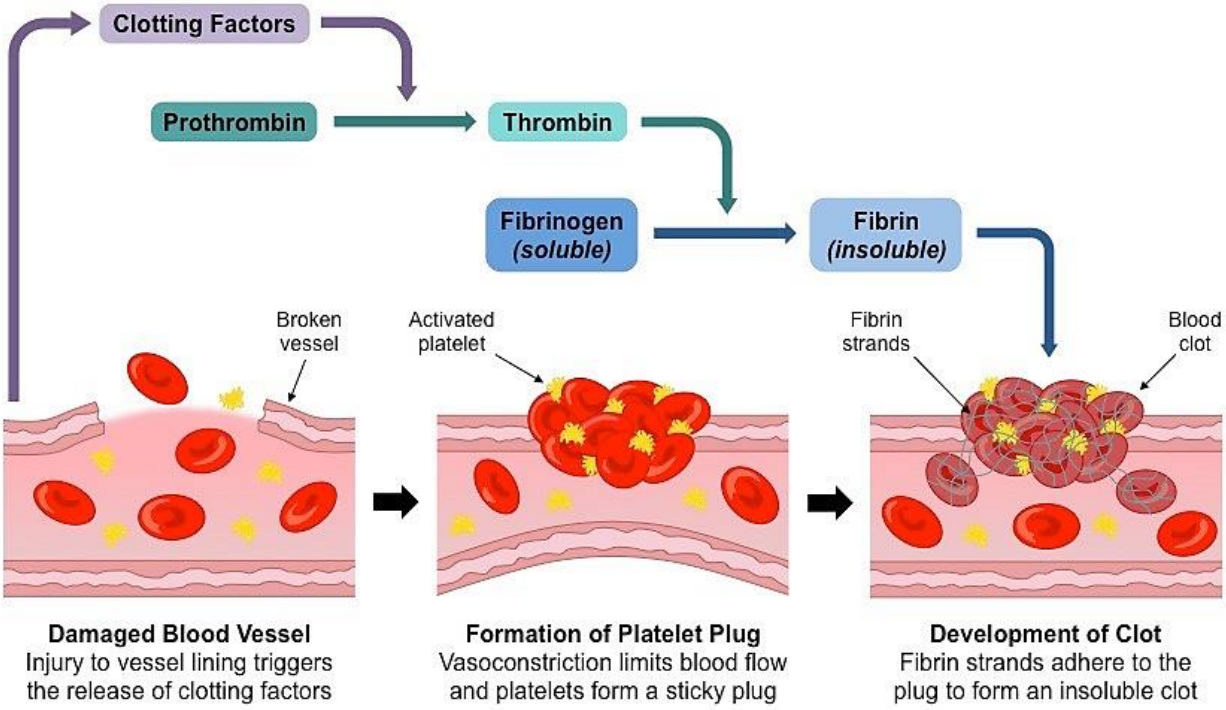


Figure 3: Coagulation cascade in response to injury.[19]

1.4.2. The inflammatory phase

The inflammatory phase often overlaps the haemostasis phase and can take up to four days.[8,17] Activation and chemotaxis occur while the fibrin clot is being formed.

Platelets secret growth factors such as, transforming growth factor beta (TGF-β), platelet derived growth factor (PDGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF) which play a vital role in the movement of monocytes and neutrophils to the wound site to begin the inflammatory phase.[8,13,16] The first cells to activate and migrate are the neutrophils which infiltrate the wound bed and initiate a cascade of tumour necrosis factor α (TNF-α) and interleukin 1β (IL-1β).[17] The latter attracts monocytes which mature into macrophages and release cytokines which stimulate other immune cells to release vasodilator prostaglandins (PG) which in turn recruit more immune cells especially neutrophils, monocytes and lymphocytes.[17]

The role of neutrophils are to clear up invasive vectors, foreign material and dead cells, whereas the role of macrophages is to secrete collagenases, matrix metaloproteases, interleukins and transforming growth factor (TGF), which stimulates the proliferation of fibroblasts, keratinocytes and smooth muscle cells.[8,17,18] Additionally, fibrin is broken down as part of the clean-up process. The degradation products, with the assistance of mast cells, also attract the cells involved in the next phase such as fibroblasts and epithelial cells.[16]

1.4.3. The proliferation phase

This phase lasts between 4 and 21 days in acute wounds.[13] During this phase, epithelialisation, angiogenesis, granulation tissue formation and collagen III deposition are the main processes which take place and are regulated by FGF, TGF-β and vascular endothelial growth factor (VEGF).[8,16] After the initial rally of neutrophils to the wound site, several other cell types are recruited to carry forward the process of repair.[3] These include monocytes but also more importantly, fibroblasts which are attracted to the wound site to initiate the proliferative phase of repair.[3]

Epithelial cells on the wound edge proliferate by sending projections into the wound gap to re-establish a protective barrier.[8] Angiogenesis occurs at the early stage and new blood capillaries sprout from nearby vessels and grow into wounds to supply nutrients to the proliferating cells.[8] The deeper portions of the clot become infiltrated by capillaries and fibroblasts and transform into a soft mass called granulation tissue.[8] Fibroblasts express keratinocyte growth factors which cause keratinocyte migration and proliferation at the wound site.[8] Keratinocytes express cytokines that cause

fibroblasts to migrate into the wound site where they produce collagen III.[8] Macrophages remove the clot while fibroblasts deposit new collagen to replace it.[8]

1.4.4. The remodelling phase

The remodelling phase overlaps the proliferation phase from day 8 and can last as long as 2 years.[13] This process involves the remodelling and realignment of the collagen tissue. The predominant cell type in this phase is the fibroblast.[16] Surface epithelial cells around the wound multiply and migrate into the wounded area beneath the scab.[8] The scab loosens and eventually falls off and the epithelium grows thicker.[6] The epithelium regenerates while the underlying connective tissue undergoes fibrosis.[6] The epithelial tissues formed by actively proliferating keratinocytes are transformed into the final barrier-forming system by terminal differentiation indicated by typical protein markers such as cytokeratins K1 and K10, involucrin and loricrin.[16] Capillaries withdraw from the wound as fibrosis progresses.[8] This phase is marked by the replacement of collagen III by collagen I deposition and restructuring to re-establish the strength of the epidermal layers.[8]

1.5. Factors affecting wound healing

Even though the wound healing process progresses naturally, an infection or the presence of free radicals can seriously delay this process by prolonging the inflammatory phase, disrupting normal clotting mechanisms, promoting disordered leukocyte function and delaying angiogenesis.[3,20]

1.5.1. The role of reactive oxygen species in wound healing

Reactive oxygen species (ROS) are oxygen associated species that have oxidative potential or higher reactivity than molecular oxygen and include: singlet oxygen, superoxide anion, hydrogen peroxide and the hydroxyl radical.[21] The ROS are produced in high amounts by the neutrophils at the wound site during the inflammatory phase along with other proteolytic enzymes.[3] The ROS and proteolytic enzymes serve as antimicrobial defences and aid in the debridement of dead tissue. However, increased numbers of neutrophils cause the release of excess ROS and proteases.[3] This overwhelms protease inhibitors which normally protect the tissue cells and the extracellular matrix resulting in oxidative damage which kills fibroblasts and other cells

and make skin lipids less flexible.[3] At high concentrations, ROS can induce severe tissue damage and even lead to neoplastic transformation decreasing the healing process by damages in the cellular membranes, deoxyribonucleic acid (DNA), proteins and lipids.[22] The effects of ROS on wounds is illustrated in Figure 4.

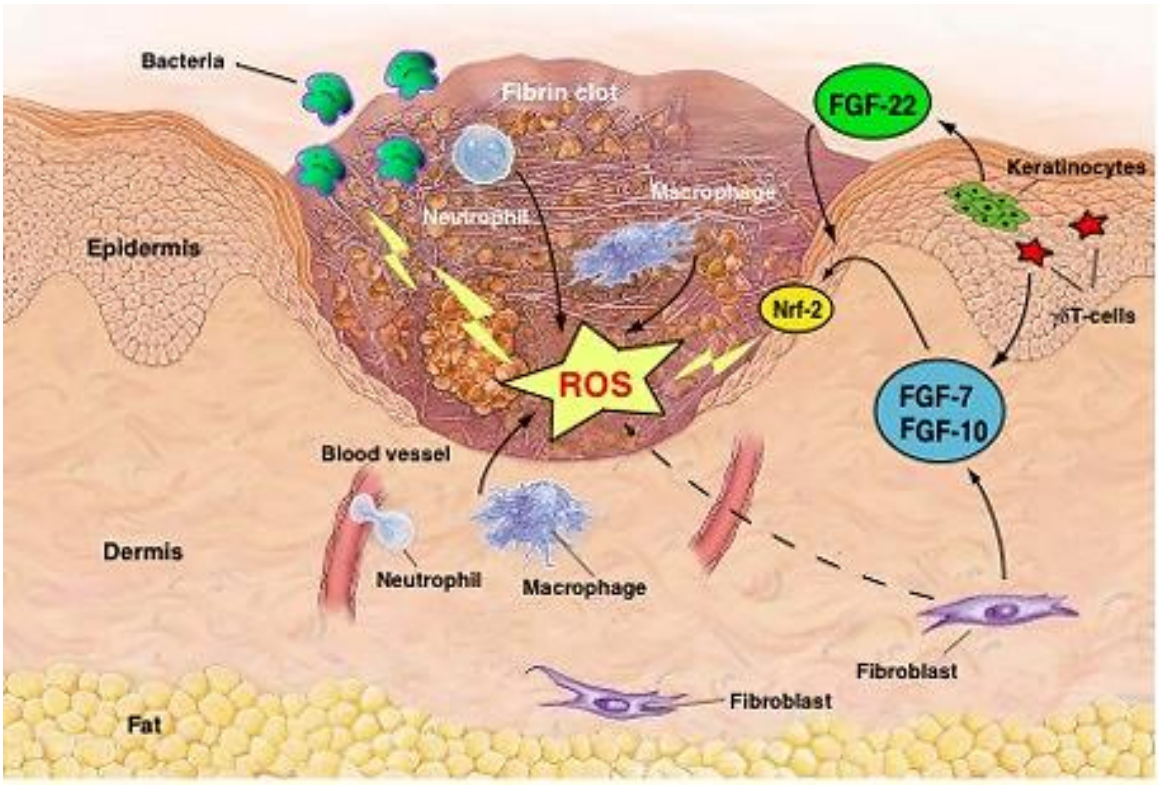


Figure 4: Effects of reactive oxygen species on wound healing.[23] NRF-2: nuclear factor erythroid 2-related factor; FGF: fibroblast growth factors.

1.5.2. The role of antioxidants in wound healing

Antioxidants appear to have a significant role in the successful treatment and management of wounds as they have the ability to reduce the adverse effects of ROS by eliminating products of inflammation.[22] Antioxidants counter the excess proteases and ROS formed by neutrophil accumulation at the wound site and protect protease inhibitors from oxidative damage.[3,22] Antioxidant protection by plant extracts or plant compounds is most likely due to the direct interaction between the extracts and the ROS which limits the damage to cell membranes.[22] Plant compounds with high radical scavenging capacity have been shown to facilitate wound healing.[22]

1.5.3. Wound healing and infection

The surface of the human body is home to approximately 1×10^{14} microorganisms that colonize it.[7] Under normal circumstances these organisms do not cause disease, but are present as the normal microbiota of the human body, however these microbiota have the potential to become opportunistic pathogens.[7] An opportunistic pathogen is a microorganism that forms part of the normal microbiota of the human body, but in a compromised patient can cause an infection.[7] A compromised patient is one whose resistance to infection has been impaired by disease, therapy or burns.[7] As long as the skin and mucous membranes remain intact, they provide formidable physical barriers against most pathogens.[7] Open wounds are particularly prone to infection by opportunistic pathogens, especially by bacteria and fungi, and provide an entry point for systemic infections.[7]

Once access is gained at the skin surface, bacteria colonize underlying tissues and cause infection within 48 h after injury.[24] Infected wounds heal less rapidly and also often result in the formation of unpleasant exudates and toxins which are produced with concomitant killing of regenerating cells.[3] The most common opportunistic pathogens include *Staphylococcus aureus*, *Enterococcus* spp, *Escherichia coli, Pseudomonas aeruginosa, Enterobacter* spp, *Clostridium difficile* and *Candida albicans.* These microorganisms result in systemic infections and are the main cause of nosocomial (hospital acquired) infections.[7]

1.5.3.1. Planktonic bacteria and biofilms

Planktonic bacteria exist in a free-floating state (Figure 5).[25] It has been proposed that the phenotypically different planktonic state of bacteria has evolved from surface attached bacteria (biofilms) as a seeding and dispersal mechanism.[25] The planktonic state of bacteria is more susceptible to antibiotics than its biofilm counterpart.[26] Although microorganisms exist in a planktonic state, it is common for them to adhere to surfaces in medical, industrial and natural settings to form biofilms (Figure 5).[27,28]

The biofilm form epitomises the predominant mode of growth for most microorganisms by enclosing them in an extracellular polymeric matrix which provides stability and protection against changes in environmental conditions such as pH, temperature and the effects of antibiotics.[28,29] Another characteristic feature of biofilms is the ability to

communicate with one another using a chemically based language termed “quorum sensing”.[28,29] The latter provides the microorganisms with a mechanism which delays the production of virulence factors enabling them to reach adequate numbers to combat host defences.[29] The protective mechanisms provided by the quorum sensing abilities of microorganisms within the biofilm has resulted in an increased resistance against antibiotics.[26,28]

Biofilms have been identified in various chronic wound environments including diabetic wounds, venous stasis ulcers, and pressure sores.[30] The presence of a biofilm on a chronic wound surface is a hindrance to normal healing and most likely constitutes its chronic state.[31] When a biofilm becomes established in an open wound, its presence becomes difficult to suppress, especially in immunocompromised patients, as the microorganisms and their extracellular components have the ability to prolong the inflammatory phase indefinitely, delaying the progression of normal wound healing.[31] In animal studies it was shown that biofilms create a low-grade and persistent inflammatory response, which impairs both epithelialization and granulation tissue formation.[25] Since there is no gold standard for biofilm treatment, management strategies of wounds include debridement, dressings and topical antimicrobials and antiseptics.[25]

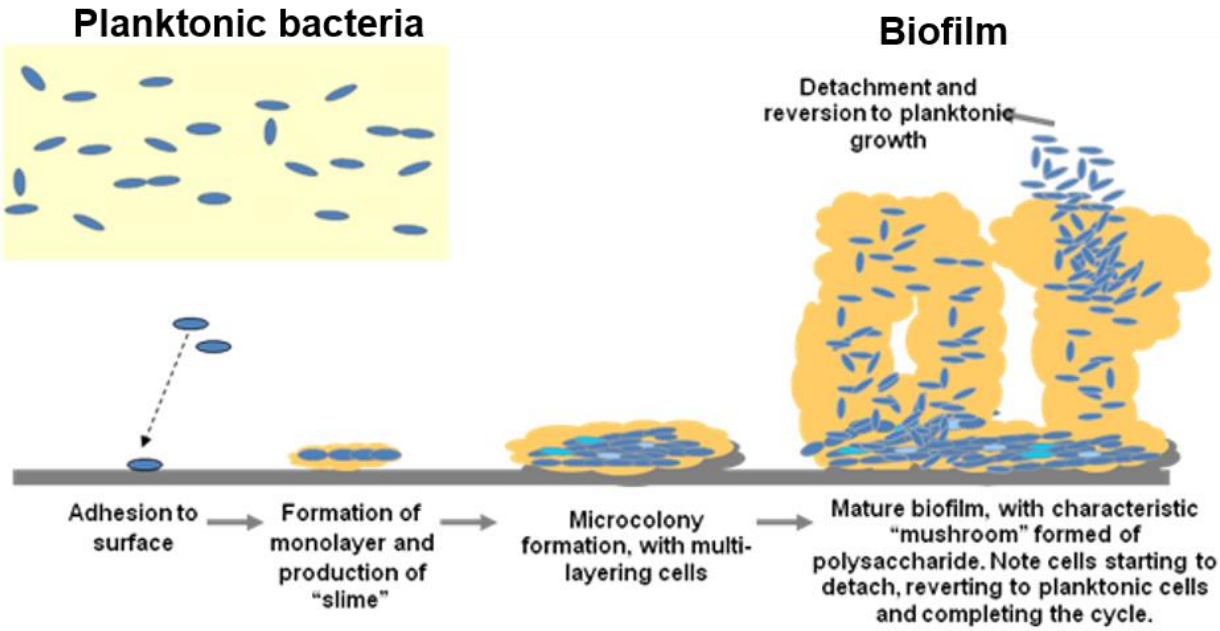


Figure 5: Progression of bacteria from a planktonic to a biofilm state.[32]

1.5.3.2. Antibiotic resistance

Antibiotic resistance has occurred as a result of decades of antimicrobial use and misuse and continues to grow as a global public health threat.[33] The World Health Organisation's (WHO) global report on surveillance of antimicrobial resistance indicates that the African region is no different to the global trend of growing antimicrobial resistance.[34] It is characterized not only by single drug resistance but as multiple drug resistance involving all major microbial pathogens and antimicrobial drugs.[33] Patients infected with resistant strains of bacteria have a higher risk of exacerbated clinical outcomes and death.[34]

The occurrence of resistance is comprised of a combination of two main components; the antimicrobial drug which is responsible for the inhibition of susceptible organisms and selection of resistant ones; and the genetic resistance determinant in microorganisms selected by the antimicrobial drug.[35] The resistant genes selected by the antimicrobial drug, propagate and are transmitted through the host to other hosts and geographical locations.[33] Genes carrying resistant traits can be transferred among bacteria of different taxonomic and ecological groups via mobile genetic elements such as bacteriophages, plasmids, naked DNA or transposons (Figure 6).[36]

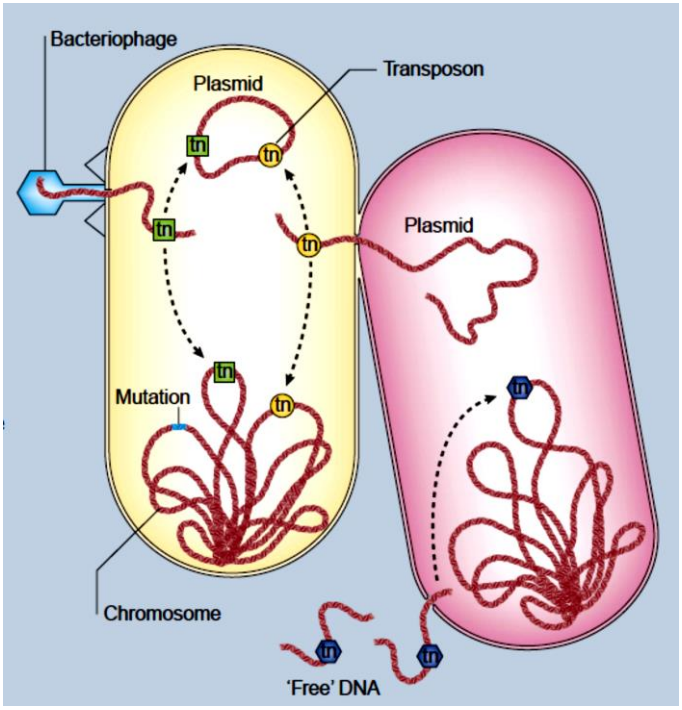


Figure 6: Transfer of resistant traits between bacterial strains.[33]

Globally, antibiotics are amongst the most purchased drugs and are used for treatment and prophylaxis of infectious diseases in humans, animals and agriculture.[37] Increased resistance to these drugs has led to the urgent need for the development of new antibiotics which have the ability to elude current resistance mechanisms.[33,34]

1.6. Models to assess wound healing

1.6.1. *In vitro* assays

New compounds are continually being isolated from traditional medicines.[3] With this, *In vitro* assays have brought about the discovery of novel therapeutic agents. Assays for wound healing exploit the different aspects in processes such as fibroblast proliferation or reduction of oxidative stress, during the different stages of the wound healing process using pharmacological agents which modulate these processes as a target (Table 1).[3]

Table 1: *In vitro* assays for the assessment of various processes involved in wound healing.

Activity	Assay	Reference
Anti-inflammatory	NFκB synthesis inhibition	Bremner *et al.*[38]
	Eicosanoid synthesis inhibition	Liao *et al.*[39]
Fibroblast proliferation	Dye uptake by viable cells	Mensah *et al.*[40]
Effect on keratinocytes	Involucerin expression	Deters *et al.*[41]
	Cytokeratin K1, K10 content	Deters *et al.*[41]
Fibroblast protein expression	Proteomics	Sampson *et al.*[42]
Collagen lattice formation	Collagen lattice contraction	Phan *et al.*[43]
Antimicrobial activity	Disk diffusion (screening)	Mensah *et al.*[44]
	Broth microdilution	Eloff[45]
Antioxidant properties	DPPH for free radical scavenging	Mensah *et al.*[46]
	ABTS for free radical scavenging	Re *et al.*[47]
	Malondialdehyde determination using thiobarbituric acid	Mensah *et al.*[40]
	Protection of growing cells challenged with oxidant	Mensah *et al.*[40]

1.6.1.1. The wound scratch assay as a measure of cellular migration

When a scratch is created in a confluent cell layer, various cellular responses are induced, such as cell proliferation, cell micro-environment interaction and cell signalling which stimulates cells to migrate into the gap.[48] This collective migration is known as sheet migration and is exhibited by epithelial, endothelial, and to some degree, fibroblast cells in many normal physiological processes such as tissue repair, embryogenesis, angiogenesis and wound healing.[48,49]

Cell migration during the wound healing process is an important step of complete healing.[50] Migration is dependent on the combined action of chemosensory stimuli, reorganization of the cytoskeleton, the formation and release of cell-matrix contacts and the local ion homeostasis across the plasma membrane.[51] The process of migration is directed when a chemotactic gradient is imposed onto cells. Platelet derived growth factors (PDGF's) serve as chemosensory regulators and chemo-attractants of migratory responses during processes such as wound healing.[51] The PDGF signals the activation of the Na^+/H^+-exchanger which controls the speed and directionality of migrating cells. In fibroblasts, the PDGF receptor, located on the cell membrane, operates via the Nck family of domain adaptors which regulates cytoskeletal reorganization and chemotaxis stimulated by PDGF.[51]

1.6.1.2. Toxicity testing

The absence of toxic effects is an important requirement for the development of a new therapeutic entity. In the decade 1991-2000, new drug registration was a mere 11% of compounds submitted for human studies, with toxicity and safety issues accounting for approximately 30% of the failures.[52] It is for this reason that possible safety issues should be detected earlier in the selection process so as to avoid wasting time and resources on the development of drugs that will later fail in clinical trials.[52]

In vitro toxicological studies are designed to detect the pharmacological hazards and establish the selectivity of new therapeutic compounds.[53] A number of assays have been developed to determine *in vitro* cytotoxicity and involve quantifying cell death/survival by assessing plasma membrane integrity, cell numeration by total protein content and enumerating viable cells through assessing certain vital

functions.[54] Popular assays that are widely used include the total cellular protein assay (sulforhodamine B), the Neutral-Red uptake assay, the lactate dehydrogenase (LDH) leakage assay, and the tetrazolium dye assays.[54]

1.6.2. *In vivo* models

In vivo models for acute wound healing generally make use of small mammals such as rabbits, guinea pigs, mice or rats due to the fact that they are easy to handle and inexpensive.[55] However, the anatomy and physiology of the skin of these animals differ vastly from the human skin.[55] Porcine models are however preferable, as the skin of the pig carries various anatomical and physiological similarities to human skin, such as: epidermal thickness, development of rete-ridges and dermal papillary bodies, abundant sub-dermal adipose tissue, similar dermal collagen, absence of the panniculus carnosus found in small loose skinned animals, size orientation and distribution of blood vessels, similar adrenexal structures, sparse body hair, functional similarities in epidermal turnover time, type of keratinous proteins, and lipid composition of the stratum corneum.[55] Also in both man and swine, partial-thickness wounds close largely though re-epithelialisation whereas small animals have a panniculus carnosus and rely on wound contraction for wound closure.[55]

Commonly used acute wound models include the incisional, excisional, dead space and burn models (Figure 7). In the incisional, excisional, and burn models, the epidermis and basement membrane are disrupted, and all wounds except burns, are filled with a provisional matrix composed of fibrin, fibronectin, and other plasma-derived components.[56] The dead space model segregates the wound space from surrounding normal tissue with a biocompatible barrier that becomes encapsulated. In the burn model, epithelial destruction is less complete in first and second degree burns, and the key feature is the residual necrotic material produced by the injury.[57] The healing process in monitored in these models by assessing the wound dimensions, epithelialization, granulation tissue and the formation of a scar.[56]

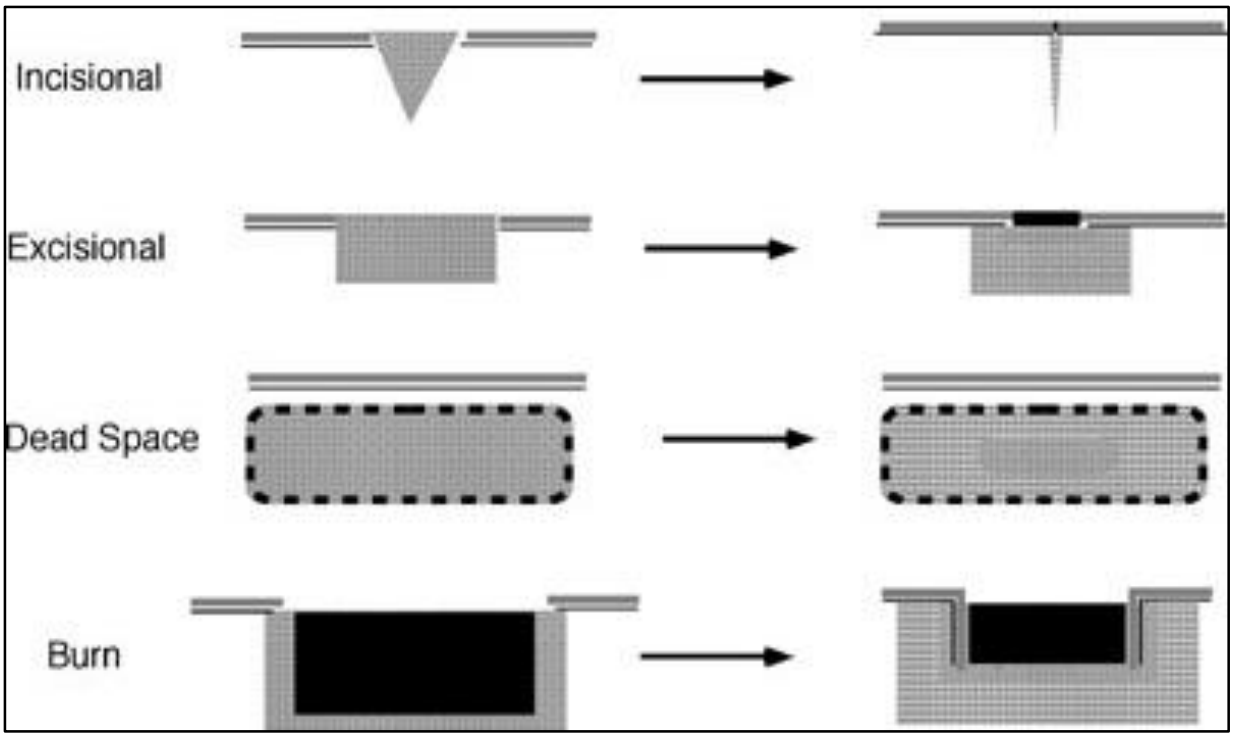


Figure 7: Cutaneous wound models.[57]

Excisional wound models involve the removal of a significant volume of the target tissue. The filling of the void created, allows for sampling of material to determine biochemical and histological parameters such as cells, tissue, ribonucleic acid (RNA), exudates, and tissue biopsies.[57] Furthermore, excisional wounds can be covered with occlusive dressings which retain the exudate (wound fluid) as a means of assessing the status of various soluble factors in the wound environment such as nutrients, proteinases, cytokines, and tissue degradation products.[45] The excisional model therefore is the preferred model for the assessment of wound healing.

1.7. Treatment of wounds

1.7.1. Western pharmaceuticals

Many classes of drugs are readily available for use as wound healing agents. The effect of a drug on a wound depends on its mechanism of action, dosage and route of administration in relation to the specific phase of wound healing.[58] Silver containing antimicrobials, such as silver sulfadiazine and silver nitrate have been considered the gold standard in wound healing for the past few decades[59] and has become routine

treatment for preventing infection in burn wounds.[60] Silver is also used in various formulations in bandages and wound dressings.[59] Silver is a broad-spectrum antimicrobial agent that kills microbes on contact through multiple mechanisms of action, has low mammalian cell toxicity and is a potent anti-inflammatory.[59] As such, silver may be used to maintain a microbe-free, moist wound healing environment.[59]

However, as with most drug classes, treatment with silver is not without limitations, which includes resistance. Treatment with silver nitrate solution, requires multiple applications per day in order to maintain a high enough concentration of silver ions available to the wound for healing.[61] This results in irritating and astringent effects as well as discolouration on tissues, poor eschar penetration has also been identified as a limitation of using silver nitrate.[61] Silver sulfadiazine cream was introduced to decrease the number of applications but also has limitations (psuedoeschar formation occurs when the cream dehydrates). Previously applied cream must first be removed before a subsequent application, which could be painful for the patient.[61] Furthermore, silver sulfadiazine has been implicated in inhibition of wound contraction required for the healing of full thickness wounds.[62] Resistance associated with silver is also an emerging issue.[59,63] There is evidence that silver-resistance genes exist sporadically in certain types of bacteria[63] creating a need for alternative agents for wound healing.

Common drug classes used in wound healing, whether primarily or off-label, include antimicrobials, methyl xanthines, retinoids, prostacyclin analogues, nitrates, calcium antagonists, systemic corticosteroids, trace minerals, anti-convulsants and analgesics (Table 2). These drugs, however, not unlike other drugs, are not without side-effects some of which can hinder the wound healing process.[64]

Table 2: Classes of drugs most commonly used in wound healing.

Drug class	Drug	Mechanism of wound healing	Indications	References
Silver containing antimicrobials	Silver sulfadiazine and silver nitrate	a) Kills microbes on contact through multiple mechanisms of action, such as inhibiting cellular respiration, denaturing nucleic acids, and altering cellular membrane permeability b) Anti-inflammatory	Topical treatment of burns and infected wounds	Warriner and Burrell[59]
Antimicrobials including iodine based preparations	Neomycin, Polymyxin B, Neosporin, Cephalosporins, Ciprofloxacin	a) Target bacteria at several level (cell membrane, cytoplasmic organelle, and nucleic acid), thus minimising bacterial resistance	Infected wounds	Karukonda *et al.* Enoch *et al.*[58,65]
Methylxanthine	Pentoxifylline	a) Improves peripheral vascular bed perfusion b) Improves capillary microcirculation by decreasing blood viscosity and reducing platelet aggregation c) May inhibit tumour necrosis factor- α an inflammatory cytokine involved in non-healing wounds	Ulcers, peripheral vascular disease, sickle cell ulcers, livedoid vasculitis, and necrobiosis lipoidica	Enoch *et al.*[65]

Drug Class	Drug	Mechanism of wound healing	Indications	References
Nitrates	Glyceryl trinitrate	a) Nitric oxide donor causing vasodilatation	Chronic wounds (ischaemic aetiology) and vasculitic ulcers	Enoch *et al.*[65]
Calcium antagonists	Diltiazem and Nifedipine	a) In Raynaud's disease, restores blood flow to the extremities and are useful in treating ulcers and the prevention of necrosis in the extremities	Vasculitic ulcers secondary to Raynaud's disease and connective tissue diseases	Enoch *et al.*[65]
Systemic corticosteroids	Prednisone	a) Promote healing by attenuating the excessive inflammatory response	Ulcers, connective tissue diseases, including rheumatoid arthritis, scleroderma, and other vasculitic disorders	Karukonda *et al.*[58] Enoch *et al.* [65]
Trace mineral	Zinc	a) DNA synthesis, cell division, and protein synthesis which are all necessary processes for tissue regeneration and repair b) Antioxidant	Used as paste on infected leg ulcers, oral zinc sulphate used to treat chronic ulcers in patients with low serum zinc levels	MacKay and Miller[66] Enoch *et al.*[65]

Drug Class	Drug	Mechanism of wound healing	Indications	References
Anticonvulsant	Phenytoin	a) Modification of collagen remodelling by inhibition of enzyme collagenase	Low grade pressure ulcers, trophic ulcers due to leprosy and dystrophic epidermolysis bullosa	Karukonda *et al.*[58] Enoch *et al.*[65] Harvey *et al.*[67]
Retinoids (derived from vitamin A)	Topical tretinoin	a) Enhances early inflammatory phase, increasing the number of monocytes and macrophages at the wound site b) Modulates collagenase activity supports epithelial cell differentiation c) Improves localization and stimulation of the immune response	Reversal of corticosteroid-induced inhibition of cutaneous and fascial wound healing, dermabraded and chemically peeled wounds, partial and full thickness wounds	MacKay and Miller [66] Enoch *et al.*[65]
Analgesics	Non-steroidal anti-inflammatory's (NSAIDs), opiates, tricyclic antidepressants (TCA's) (amitriptyline) or antiepileptics (gabapentin)	a) NSAIDs- dose dependent effect on inflammation b) Opiates- alleviation of pain by altering perception of pain c) TCA's- inhibit the reuptake of the biogenic amines d) Antiepileptics- affect voltage dependent calcium ion channels at postsynaptic dorsal horn interrupting events leading to neuropathic pain	Ulcers, neuropathic pain from ulcers	Karukonda *et al.*[58] Rose and Kam[68] Enoch *et al.*[65] Harvey *et al.*[67]

1.7.2. Medicinal plants

Traditional therapies, such as herbal remedies, are used primarily as healthcare in approximately 80% of the population in African countries.[69] Traditional medicine is embedded in the roots of African culture and forms a fundamental part of many indigenous beliefs. More importantly, it is utilised by the indigenous population due to limited access to conventional healthcare and the high costs associated with it.[70] In South African rural areas, traditional healers are the first health providers to be consulted in the majority of cases.[71]

Traditional medicines are used for the treatment of a wide range of conditions, including, acute and chronic medical conditions, supernatural or psychosocial problems, acute conditions, generalized pain, human immunodeficiency virus (HIV) and other sexually transmitted infections as well as for wound healing.[72] The use of plants and preparations thereof to accelerate the wound healing process can be traced back to ancient times.[73] There are many controversies surrounding the folkloric uses of herbal remedies for the treatment of disease.[74] *In vitro* testing has provided a means for scientifically validating ethnomedicinal usage of plants and eliminating controversies.[74]

Compounds present in plants have be proven to be more effective than conventional treatments, providing wider treatment options to modern medicine, examples of such compounds include digitoxin, rutin, morphine, codeine, atropine, scopolamine, quinine and reserpine to mention a few.[74] Conversely, it is also important to determine which plants/compounds are safe for use in order to avoid harmful effects and so that a protocol can be put in place in case of poisoning.[74,75] It is for these reasons that validation of herbal remedies for ethnomedicinal treatment of disease requires pharmacological studies.

A plethora of African medicinal plants from various plant families and species have been reported as being used in ethnomedicinal wound care. All African plants tested for wound healing activity have been compiled in a recent review by Agyare and co-workers.[16] These have been summarized in Table 3. The plants listed in this review were all scientifically validated for their ethnomedicinal usage in wound healing, this includes a total of 61 plants from 36 families.

Table 3: African medical plants used in wound healing (Summarized with permission from Agyare *et al.*[16])

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Acanthaceae	*Justicicia flava* (Forssk.) Vahl	Coughs, paralysis, fever, epilepsy, convulsion and spasm, and skin infections and disorders, as well as diarrhoea and dysentery.	Methanol leaf extract (7.5% w/w)	• Reduction in wound size in rat excision wound model • Increase tensile strength of wounds • Improved angiogenesis, collagenation, re-epithelialisation in animal model	Burkill [76] Agyare *et al.*[77] Agyare *et al.*[78]
Amaranthaceae	*Alternanthera sessilis* (L.) R. Br. ex DC	Ulcers, cuts, wounds, fevers, ophthalmia, gonorrhoea, pruritis, burning sensations, diarrhoea, skin diseases, dyspepsia, haemorrhoids, liver and spleen diseases.	Chloroform leaf extract (200 mg/kg)	• Reduction in wound area and increased re-epithelialisation at dosage 200 mg/kg • Complete epithelialisation and improved wound breaking strength in excision model	Jalalpure *et al.*[79] Hossain *et al.*[80]
	Pupalia lappacea (L.) Juss.	Boils, chronic wounds and skin infections, leaf paste used to treat bone fractures and inflammatory conditions.	Ethanol leaf extract (2% and10% w/v cream) and Chloroform leaf extract (1% w/v cream)	• Substantial collagenation, re-epithelialisation, granular tissue formation and angiogenesis noted in histological studies • Wound closure in rat excision wound model	Agyare *et al.*[77] Ravi *et al.*[81] Neeharika *et al.*[82] Apenteng *et al.*[83]
			Methanol leaf extract (20% w/v ointment)	• Acceleration of wound healing and significantly higher wound breaking strengths and weight of granuloma tissues	Udegbunam *et al.*[84]

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Anacardiaceae	*Lannea welwitschii* (Hiern) Engl.	Diarrhoea, dysentery, swellings, gout, gingivitis, topical infections, and wounds and roots are used for food poisoning, nasopharyngeal infections and as emetics.	Methanol leaf extract (7.5% w/w)	• Reduced wound size in rat excision wound model • Increased the tensile strength of wounds • Wound tissues showed improved angiogenesis, collagenation, and re-epithelialisation	Agyare *et al.*[77,24,78]
Apiceae	*Cuminum cyminum* L.	Seeds used for culinary and flavouring purposes and folklore therapy.	Alcoholic seed extract and petroleum ether extract	• Re-epithelialisation • Promoted wound healing activity in excision, incision and granuloma wound models	Rechinger[85] Mozaffarian[86] Patil *et al.*[87] Gohari and Saeidnia[88]
Apocyanaceae	*Catharantheus roseus* L.	Applied as a paste on wounds, fresh juice made into a tea for external use to treat skin problems, dermatitis, eczema and acne.	Ethanol flower extract.	• Increased wound breaking strength incision wound model • Faster epithelialisation and significantly increased rate of wound contraction • Increase in wet and dry granulation tissue weights and hydroxyproline content in a dead space wound model	Nayak and Pereira[89]
	Strophanthus hispidus DC	Antidote for snake bites, syphilis ulcers, bony syphilis, guinea worm sores and wounds.	Leaf and root extracts (7.5% w/w)	• Wound contraction • Improved collagenation, re-epithelialisation and rapid granulation formation	Krasner *et al.*[90] Agyare *et al.*[24]

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Apocyanaceae	*Wrightia tinctoria* R. Br.	Various diseases	Ethanol stem bark extract	• Improved breaking strength • Increased percentage wound closure and decreased epithelialisation time • It also increased breaking strength and hydroxyproline content in dead space wound model	Joshi *et al.*[91] Singh *et al.*[92] Shah and Gopal[93] Veerapur *et al.*[94]
Asclepiadaceae	*Calotropis gigantean* R. Br.	Skin diseases, boils, sores and wound healing in combination with other plants.	Whole plant extract	• Increased percentage of wound contraction in scar area • Decreased re-epithelialisation time • Improved breaking strength and hydroxyproline content	Chitme *et al.*[95] Ahmed *et al.*[96] Argal and Pathak[97] Deshmukh *et al.*[98]
	Calotropis procera (Aiton) Dryand.	Wound healing	Latex extract (1% *w/v*)	• Increased collagen, DNA and protein synthesis and epithelialisation • Reduction in wound area in excision wound model.	Rasik *et al.*[99]
Asteraceae	*Ageratum conyzoides* L.	Application to fresh wounds and burns.	Methanol and aqueous leaf extracts	• Faster rate of wound healing in excision, incision and dead space wound models	Watt and Breyer-Brandwijk[100] Oladejo *et al.*[101]
			Chloroform leaf extract	• Promising results but to lesser extent than methanol and aqueous extracts	
	Chromolaena odorata L.	Treatment of wounds	Crude ethanol extract	• Antioxidant protection of fibroblasts and keratinocytes *in vitro*. • Protection of cultured skin cells against oxidative damage in colorimetric and lactate dehydrogenase release assay	Phan *et al.*[102]

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Asteraceae	*Centaurea iberica* Trev. ex Spreng	Alleviation of pain and inflammatory symptoms in rheumatoid arthritis, high fever, headache and wounds.	Aqueous (ointment) and methanol extracts	• Supported outcome incision and excision wound models • Methanol extract exerted remarkable wound healing activity and demonstrated a significant dose-dependent anti-inflammatory activity.	Koca *et al.*[103]
	Sphaeranthus indicus L.	Gastric disorder, skin diseases, anthelmintic, glandular swelling, nervous depression, bronchitis, asthma, leucoderma, jaundice and scabies.	Aerial extract	• Significantly enhanced the rate of wound contraction and the period of epithelialisation comparable to neomycin in pigs	Chopra *et al.*[104] Nadkarni[105] Kirtikar and Basu[106] Sadaf *et al.*[107]
	Tridax procumbens L.	Wound healing	Whole plant and aqueous extract	• Increase in lysyl oxidase activity, protein and nucleic acid contents as well as the tensile strength in dead space animal model	Udupa *et al.*[108] Yaduvanshi *et al.*[109] Talekar *et al.*[110]
			Juice (1µg/mL)	• Increase in collagen biosynthesis • Increased infiltration of inflammatory cells, fibroblast proliferation and re-epithelialisation with moderate vascularity in dermal wounds	
			Ethanolic and aqueous extract	• Significant (p< 0.05) increase in wound tensile strength. • Biochemical markers hydroxyproline, collagen and hexosamine increased significantly (p<0.05) in excision model	

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Bignoniaceae	*Kigelia africana* (Lam.) Benth.	Skin ailments including fungal infections, boils, psoriasis and eczema as well as leprosy, syphilis and cancer.	Methanol stem bark extract (7.5% w/w aqueous cream)	• Wound contraction in rat excision wound model • Improved collagenation, re-epithelialisation and rapid granulation formation	Houghton and Jâger[111] Agyare *et al.*[24]
	Spathodea campanulata P.Beauv.	Stem bark is applied to wounds in form of a paste.	20% w/w *Spathodea* cream	• Excision wounds treated with *Spathodea* cream in combination with Cicatrin® cream showed a rapid and comparable decrease in wound size in rats	Mensah *et al.*[44] Houghton *et al.*[3] Ofori-Kwakye[112]
Boraginaceae	*Heliotropium indicum* L.	It is used as an analgesic (rheumatism), diuretic and for numerous skin problems (e.g. yaws, urticarial, scabies, ulcers, eczema, impetigo and wounds).	Methanol extract	• Increase in granulation tissue weight, increased hydroxyproline content and increased activity of superoxide dismutase and catalase level in dead space animal wound	Dash and Murthy[113] Iwu[114] Sofowara[115] Burkill[116,117]
Cactaceae	*Opuntia ficus-indica* (L.) Mill.	Treatment of various diseases including inhibition of stomach ulceration.	Methanol stem extract and its hexane, ethyl acetate, *n*-butanol and aqueous fractions	• Methanol extract and less polar fractions showed significant wound healing effects	Park and Chun[118] Galati *et al.*[119] Trombetta *et al.*[120]
			Polysaccharide extracts	• Polysaccharides with a molecular weight accelerated the re-epithelialisation and remodelling phases, also by affecting cell-matrix interactions and by modulating laminin deposition	

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Caricaceae	*Carica papaya* L.	Wound dressing	Aqueous leaf extract (5 and 10% w/v in vaseline)	• Accelerated wound healing in excision and dead space wound models • Wet and dry granulation tissue weight and hydroxyproline content increased significantly	Burkill[121] Mahmood *et al.*[122] Nayak *et al.*[123] Gurung and Škalko-Basnet[124]
			Dried papaya latex (1% and 2.5% in carbopol gel)	• Accelerate wound closure, increase hydroxyproline content and stimulate epithelialisation in burn wound model	
Cecropiaceae	*Myrianthus arboreus* P. Beauv	Dysentery, diarrhoea, wounds, boils, dysmenorrhoea, incipient hernia and vomiting.	Methanol leaf extract (5% w/w cream)	• Potent wound healing capacity with better wound closure on day 1 and day 9 • Enhanced wound tissue proliferation, fibrosis and re-epithelialisation	Agyare *et al.*[125]
Combretaceae	*Terminalia arjuna* (Roxb. ex DC) Wight and Arn.	Management of dysentery and rheumatism	Fractions from hydro-alcoholic extract of the stem bark	• Fractions significantly increased the tensile strength of the incision wounds and degree of re-epithelialisation of excision wounds in animals	Chaudhari and Mengi[126]
	Combretum mucronatum Schum and Thonn.	Wound healing	Aqueous leaf extract (1 and 10 µg/mL)	• Stimulates viability of human keratinocytes and dermal fibroblasts. • Significantly stimulated cellular differentiation of primary keratinocytes	Kisseih *et al.*[127]
Curcubitaceae	*Momordica charantia* L.	Management of wounds, peptic ulcer, fever, piles and skin infections, parasitic infections and treatment of yaws.	Methanol leaf extract	• Wound closure in rat excision wound model • Histological investigation revealed high fibrosis and collagenation	Burkill[128] Agyare *et al.*[77,129]

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Euphorbiaceace	*Alchornea cordifolia* (Schum. &Thonn.) Muell. Arg.	Treatment of wounds, leprosy and as antidote to snake venom.	Aqueous leaf extract (10% w/w cream)	• Exhibited potent wound healing capacity with better wound closure at day 1 and day 9 in excision wound model • Histological investigations showed enhanced wound tissue proliferation, fibrosis and re-epithelialisation	Burkill [130] Agyare *et al.*[77,125]
Euphorbiaceace	*Jatropha curcas* L.	Management of ulcers and sores	Leaf and stem bark	• Accelerates the healing process by increasing the skin breaking strength, granulation tissue breaking strength, wound contraction, dry granulation tissue weight and hydroxyproline levels • Histopathological examination of granulation tissue displayed presence of collagen organised into bundles indicative of advanced wound healing	Goonasekera *et al.*[131] Igoli *et al.*[132] Shetty *et al.*[133] Zippel *et al.*[134]
			Arabinogalactan protein JC isolated from *Jatropha curcas* seed endosperm (10 and 100 µg/mL)	• Stimulated mitochondrial activity of keratinocytes and dermal fibroblasts and the ATP status of primary keratinocytes. • Triggered primary keratinocytes into differentiation status. • *In vitro* activity profile indicated JC to be a potent inducer of cellular differentiation via stimulation of growth hormones and TGF-β-induced cell signalling	
	Mallotus oppositifolius (Geiseler) Müll. Arg.	Tapeworms, diarrhoea, cuts, sores, skin eruptions and rashes, burns, headaches, epilepsy, tooth ache and inflamed eyes.	Methanol leaf extract	• Significant wound closure, fibrosis and collagenation in excision wound model	Burkill[128] Iwu[114] Kabran *et al.*[135] Agyare *et al.*[129]

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Gentianaceae	*Anthocleista nobilis* G. Don	Curing fever, stomach ache, diarrhoea, and gonorrhoea and as poultice for sores.	Methanol extract (33.3% w/w)	• Enhanced wound closure and • hydroxyproline production in excision wound model in rats • Significant increase in tensile strength in incision wound model	Dokosi[136] Irvine[137] Annan and Dickson[138]
Hypericaceae	*Hypericum patulum* Thunb.	It is used traditionally to treat dog bites and bee stings.	Methanol leaf extract (5% and 10% w/w ointment)	• Enhanced wound contraction rate, re-epithelialisation, tissue granulation and increased tensile strength in excision and incision model of wounds in rats	Baruah *et al.*[139] Mukherjee *et al.*[140]
	Hypericum perforatum L.	Treatment of wounds	Total extract	• Improved wound contraction rate and period of re-epithelialisation in linear incision, circular excision and thermal burn	Süntar *et al.*[141] Prisăcaru *et al.*[142] Mainetti and Carnevali[143] Laeuchli *et al.*[144]
			Ointment	• Significant wound healing effect in skin injuries and appears to be safe for use	
			Wound dressing containing a mixture of hypericum oil (*Hypericum perforatum* L.) and neem oil (*Azadirachta indica* A. Juss.)	• Enhances granulation tissue formation and re-epithelialisation in scalp wounds with exposed bone and paediatric burn wounds	

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Lamiaceae	*Occimum sanctum* L.	Infections and skin diseases	The alcoholic extract (400 mg/kg) and aqueous extract (800 mg/kg)	• Increased wound contraction rate, wound breaking strength, hydroxyproline, hexuronic acid, hexosamines, superoxide dismutase, catalase and reduced glutathione levels in wound excision and incision models	Godhwani *et al.*[145] Shetty *et al.*[146] Goel *et al.*[147]
			Aqueous leaf extract (10% in petroleum jelly)	• Lipid peroxidation was reduced • Increased rate of wound contraction and reepithelialisation in excision model	
	Occimum gratissimum L.	The leaves are rubbed between the palms and sniffed as a treatment for blocked nostrils.	Leaf extract	• Significant wound contraction • Histology of the healed scar showed non-significant decrease in the mean fibroblast count	Osuagwu *et al.*[148] Chah *et al.*[149]
	Hoslundia opposita Vahl	Sore throats, colds, sores, veneral diseases, herpes, skin diseases, malaria, microbial infections, epilepsy, fever and inflammation.	Methanol extract (33.3% w/w)	• Increased wound contraction and hydroxyproline content excision wound model in rats • Significantly improved tensile strength of wounds in incision wound model	Abbiw [150] Olajide *et al.*[151] Moshi and Mbwambo[152] Annan and Dickson[138] Agyare *et al.*[16]

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Lamiaceae	*Hyptis suaveolens* (L.) Poit.	Treatment of various diseases and the essential oil has been found to possess insecticidal and larvicidal properties.	Ethanol leaf extract (400 and 800 mg/kg)	• Increased skin breaking strength, granuloma breaking strength, wound contraction, hydroxyproline content and dry granuloma weight and decreased the re-epithelialisation period • Increase in catalase and superoxide dismutase levels of granuloma tissue	Peerzada[153] Shirwaikar *et al.*[154] Shenoy *et al.*[155]
			Petroleum ether, alcohol, and aqueous leaf extracts (500 mg/kg)	• Acceleration of wound closure and period for re-epithelialisation in rat excision model • Increased tensile strength, dry weight granulation tissue, breaking strength of granulation tissue and hydroxyproline content in incision and dead space models	
	Leucas hirta (B. Heyne ex Roth)	Skin diseases, thrush, snake bites, swelling and boils, cure eye sores.	Aqueous and methanol leaf extracts (35 mg/kg)	• Decrease in re-epithelialisation period in excision wound model • Increased rate of wound contraction, skin breaking strength, granulation tissue dry weight, hydroxyproline content and breaking strength of granulation tissue in incision and dead space wound models • Histopathological study of granulation tissue showed increased collagenation	Williamson[156] Manjunatha *et al.*[157]

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Lythraceae	*Lawsonia inermis* L.	Wound healing	Ethanol leaf extract (200 mg/kg/day)	• Significantly enhanced wound contraction, increased skin breaking strength, hydroxyproline content and significant decrease in the period of epithelialisation	Nayak *et al.*[158]
	Punica granatum L.	Wound healing	Leaf extract (2.5% and 5% w/w)	• Enhanced wound healing activity in rats • Increased hydroxyproline content	Chidambara Murthy *et al.*[159]
Malvaceae	*Hibiscus rosa sinensis* L.	Variety of diseases and wound healing	Ethanol leaf extract (120 mg/kg/day)	• 86% reduction in the wound area in excision rat model • Significant epithelialisation in incision model • Significantly increased skin-breaking strength • Significantly increased hydroxyproline content in dead space model	Nayak *et al.*[160] Bhaskar and Nithya[161]
			Ethanolic extract of the flowers (5% and 10% w/w)	• Increased cellular proliferation and collagen synthesis at the wound site • Promotes wound healing • Improved rates of epithelialisation, wound contraction as well as increased wound tensile strength and wet and dry granulation tissue weights	

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Meliaceae	*Carapa guianensis* Aubl.	Ulcers, skin parasites, and skin problems.	Ethanol leaf extract	• 100% reduction in the wound area when compared to controls (95%) with • Significant decrease in the epithelialisation period • Significantly increased skin breaking strength, wet and dry granulation tissue and hydroxyproline content	Nayak *et al.*[162]
	Azadirachta indica A. Juss	Wound healing	Aqueous leaf extract	• Significantly increased rate of wound closure in excision wound model	Barua *et al.*[163] Pandey *et al.*,[164] Osunwoke Emeka *et al.*[165]
			Methanol leaf extract (5% w/w ointment)	• Promotes wound healing activity in both excision and incision wound models	
			Oil	• Increased wound tensile strength and wound closure rate	
Moringaceae	*Moringa oleifera* Lam.	Psychosis, eye diseases, fever as an aphrodisiac wound management.	Ethanolic and ethyl acetate seed extracts (10% ointment)	• Significant antipyretic activity in rats Significant wound healing activity in excision, incision and dead space (granuloma) wound models in rats	Chopra[166] Hukkeri *et al.*[167]
Musaceae	*Musa sapientum* L.	Ulcers	Aqueous and methanol extracts	• Increased wound breaking strength and levels of hydroxyproline, hexuronic acid, hexosamine, superoxide dismutase, reduced glutathione in the granulation tissue • Decreased percentage of wound area, scar area and lipid peroxidation	Goel and Sairam[168] Agarwal *et al.*[169]

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Myrsinaceae	*Embelia ribes* Burm.	Toothache, headaches, snakebite, maintaining healthy skin, support of digestive function, treatment of fever, abdominal disorders, lung diseases, constipation, fungal infections, mouth ulcer, sore throat, pneumonia, heart disease and obesity.	Embelin- Isolated quinone compound from ethanol leaf extract (4 mg/mL in a 0.2% w/v sodium alginate gel)	• Rapid re-epithelialisation of incision wound with a high rate of wound contraction • Significantly increased tensile strength of incision wound • In dead space wound model, increased weight of the granulation indicating an increase in collagenation • Histology showed increased cross-linking of collagen fibres and absence of monocytes	Chopra *et al.*[170] Swamy *et al.*[171] Lal and Mishra[172]
Pedaliaceae	*Sesamum indicum* L.	Fever, cough, sore eyes, dysentery, gonorrhoea, ulcers and treatment of various kinds of wounds.	Seeds (2.5% w/w) and oil treatment (5% w/w)	• Significant decrease in the period of epithelialisation and wound contraction • Increased breaking strength of wound tissues • Significant increase in the breaking strength, dry weight and hydroxyproline content of the granulation tissue in dead space wound model	Kiran and Asad[173]
Piperaceae	*Piper betel* L.	Wound healing	Dried residue of aqueous extract (1% ointment in soft paraffin)	• Found to possess wound healing activity	Santhanam and Nagarajan[174]
Potulacaceae	*Portulaca oleracea* L.	External treatment of ulcers, eczema and dermatitis.	Crude aerial parts (50 mg, followed by 25 mg treatment)	• Accelerated the wound healing process in mice • Decreased the surface area of wound and increased the tensile strength treatment	Rubatzky and Yamaguchi[175] Rashed *et al.*[176]

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Phyllanthaceae	*Bridelia ferruginea* Benth	Bruises, boils, burns, wounds and skin disease.	Ethanol stem bark extract (1–30 µg/mL)	• Enhanced wound contraction and epithelialisation • Influence on the proliferation of dermal fibroblasts	Sofowora[115] Udegbunam *et al.*[177] Adetutu *et al.*[178]
Rubiaceae	*Morinda citrifolia* L.	Topical treatment for wound healing	Ethanol extract of leaves (150 mg/kg/day)	• Reduced wound area in excision wound model • Decreased epithelialisation time • Increase in granulation tissue weight and hydroxyproline content in dead space wound model	Nayak *et al.*[179,180]
	Pentas lanceolata (Forssk.) Deflers	Wound healing	Ethanol extract of flowers (150 mg/kg)	• Significant increment in granulation tissue weight, tensile strength, hydroxyproline and glycosaminoglycan content in excision wound model • Marked increase in the wound contraction and collagen deposition	Nayak *et al.*[181]
	Rubia cordifolia L.	Skin diseases such as eczema, dermatitis and skin ulcers.	Alcoholic extract (hydrogel)	• Improved wound contracting ability, wound closure, decrease in surface area of wound, tissue regeneration at the wound site significantly in mice	Karodi *et al.*[182]
Sapotaceae	*Mimusops elengi* L.	Headache, toothache, wounds, sore eyes, infections of the nose and mouth, fever, diarrhoea, inflammation of the gums, toothache, gonorrhoea and diarrhoea.	Methanol extract of bark (5% w/w)	• Significant influence on the rate of wound closure, tensile strength and dry granuloma weight	Lemmens[183] Pennington[184] Shah *et al.*[185] Gupta and Jain[186]

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Solanaceae	*Datura metel* L.	Haemorrhoids, boils, sores and skin diseases.	Ethanol leaf extract	• Increased cellular proliferation and collagen synthesis at wound site • Increased rates of epithelialisation and wound contraction • Increase in wound breaking strength • Increased rate of wound contraction • Wet and dry granulation tissue weights increased significantly • Significant increase in wound closure rate, tensile strength, dry granuloma weight and wet granuloma weight	Avery *et al.*[187] Christian[188] Nithya[189]
	Solanum xanthocarpum Schrad and H. Wendl.	Bronchial asthma, cough, worms, to facilitate the seminal ejaculation, itching,fever and reduce fats.	Ethanol leaf extract	• Improved wound healing via increased re-epithelialisation, tensile strength and hydroxyproline content	Dewangan *et al.*[190]
Verbenaceae	*Clerodendrum splendens* G. Don	Vaginal thrush, bruises, wounds and various skin infections.	Methanolic extract of the aerial parts	• Improved wound closure and hydroxyproline biosynthesis compared in the excision wound model in rats • Increased the tensile strength of wounds	Irvine[137] Gbedema *et al.*[191]
	Lantana camara L.	Skin itches, antiseptic for wounds and externally for leprosy and scabies.	Leaf extract	• Enhanced the rate of wound contraction (98%), synthesis of collagen and decreased mean wound healing time.	Lyons and Miller[192] Nayak *et al.*[179,180]
Zingiberaceae	*Curcuma aromatica* Salisb.	Stomachic, carminative and emmenogogue remedies for skin diseases and snakebites.	Ethanol extract of dried rhizome (1% ointment in soft white paraffin)	• Promotes wound contraction and epithelialisation • Significantly improved wound contraction in rat excision wound model	Chopra *et al.*[193] Santhanam and Nagarajan[174] Kojima *et al.*[194] Kumar *et al.*[195]

Family	Plant	Ethnomedicinal usage	Plant part and extraction	Wound healing abilities	Reference
Zygophyllaceae	*Balanites aegyptiaca* L. Diel.	Circumcision wounds, worm infestation, abortifacient, contraceptive and abdominal and chest pains.	Methanol extract (33.3% w/w)	• Significantly improved wound closure and hydroxyproline progfduction in the excision wound model in rats • Significantly increased tensile strength	Liu and Nakanishi[196] Kurokawa *et al.*[197] Annan and Dickson[138]
	Fagonia schweinfurthii (Hadidi) Nabil and Hadidi	Inflammation, open wounds, boils, skin Eruptions and allergies.	Ethanol extract Formulated gel (10% and 20%)	• Enhance wound closure time in excision wound model in rats	Alqasoumi *et al.*[198]

From the review, the most common plant part used in wound healing was found to be the leaves, while methanol and ethanol were the most commonly used extract solvents and also exhibited the most promising effects on wound healing.

In vivo murine wound models were the predominant method used for the assessment of wound healing and included excision, incision, dead space and burn wound models (Table 3). The *In vivo* models were utilised for the evaluation of rate of wound closure (contraction), tensile strength or breaking strength determination, antioxidant and antimicrobial activities, hydroxyproline content and histological investigations including epithelialisation, collagen synthesis, and granulation tissue formation. The *In vitro* studies, assessed proliferation and differentiation by monitoring differentiation markers such as collagen and keratin in mostly fibroblast and keratinocyte cell lines.

1.7.3. Phytochemicals responsible for wound healing

The bioactivity of medicinal plants is attributed to the active phytochemicals contained within them. The activity of the active phytochemical in wound healing can be summarised under the following categories: phytochemical constituents contributing to antimicrobial activity, phytochemicals working as antioxidants and as free radical scavengers and phytochemicals enhancing cell proliferation, angiogenesis, collagen production and DNA synthesis.[199] The phytochemicals responsible for wound healing in medicinal plants have been reviewed by Ghosh and Gaba (2013)[199] and are summarised in Table 4.

Table 4: Phytochemicals with wound healing properties (Summarised from Ghosh and Gaba[199])

Phytochemical class	Phytochemicals	Plant	Bioactivity	Reference
Flavonoids	Hesperidin, kaempferol, luteolin, naringenin, patuletin, quercetin, quercetagetin, vestitol	*Allamanda cathartica, Artemisia absinthium, Coronopus didymus. Cuminum cyminum, Flaveria trinervia, Heliotropium indicum, Hippophae rhamnoides, Ipomoea carnea, Jatropha curcas, Lawsonia alba, Litsea glutinosa, Rosmarinus officinalis, Moringa oleifera, Olea europaea, Pedilanthus tithymaloides, Sambucus ebulus, Scorzonera spp.*	• Scavengers of ROS • Antioxidant properties • Antimicrobial activity	Reddy *et al.*[201] Stevenson *et al.*[202] Fu *et al.*[203] Nayak *et al.*[204] Umadevi *et al.*[205] Rathi *et al.*, [206] Hukkeri *et al.*[167] Shetty *et al.*[133] Ambiga *et al.*[207] Marwah *et al.*[208] Upadhyay *et al.*, 2009[209] Patil *et al.*[87] Sriwiroch *et al.*[210] Süntar *et al.*[211] Devi and Meera, [212] Abu-Al-Basal[213] Dodehe *et al.*[214] Dash and Murthy[113] Upadhyay *et al.*[215] Sachdeva *et al.* [216] Nithya and Baskar[217] Gong *et al.*[218] Koca *et al.*[219] Craciunescu *et al.*[220] Kalirajan *et al.*[221] Sutar *et al.*, [222]
Water soluble alkaloids	Quinazolines, isoquinazolines and indole derivatives: betalains and eumelanins	*Adhatoda vasica, Adhatoda zeylanica, Berberis lycium, Catharanthus roseus*	• Antioxidant and antimicrobial properties	Bhardwaj and Gakhar[223] Nayak and Pereira[89] Vinothapooshan and Sundar[224] Shabbir *et al.*[225]

Phytochemical class	Phytochemicals	Plant	Bioactivity	Reference
Anthocyanins	Aurantinidin, cyanidin, delphinidin, europinidin, luteolinidin, malvidin, pelargonidin, peonidin, petunidin, rosinidin	Black soyabean seed coat, *Anadenanthera colubrine, Caralla brachiata*	• Radical scavengers • Antibacterial activity • Enhanced wound healing properties	Pessoa *et al.*[226] Xu *et al.*[227]
Quinones	Alkannin, alpha-methylbutylalkannin, teracrylalkannin, beta-hydroxyisovalerylalkannin, beta-acetoxyisovaleryalkannin, shikonin, beta-hydroxyisovalerylshikonin, deoxyshikonin	*Alkanna tinctoria, Arnebia densiflora, Arnebia euchroma.*	• Antimicrobial properties • Antioxidant properties	Ogurtan *et al.*[228] Kosger *et al.*[229] Pirbalouti *et al.*[230]
Terpenoids	Mono-cyclic and multi-cyclic terpenoids	*Achillea biebersteinii, A. millefolium, A. oxyodonta, A. setacea and A. teritifolia, A. vermicularis, Achyranthes aspera, Allamanda cathartica, Alternanthera sessilis, Anredera diffusa, Arnebia densiflora, Berberis lycium, Caesalpinia benthamiana, Celastrus paniculatus, Centella asiatica, Cissus quadrangularis, Croton bonplandianum, Croton stellatopilosus Ohba, Elephantopus scaber, Heliotropium indicum, Laurus nobilis, Paullinia pinnata, Vernonia arborea Hk.*	• Antimicrobial activity • Manifest antimicrobial effects through synergy with other compounds present in the plant extract	Maquart *et al.*[231] Ünlü *et al.*[232] Singh *et al.*[233] Manjunatha *et al.*[234] Bariş *et al.*[235] Nayak *et al.*[204] Moura-Letts *et al.*[236] Asif *et al.*[237] Dickson *et al.*[238] Harish *et al.*[239] Jalalpure *et al.*[240] Kosger *et al.*[229] Mohanty *et al.*[241] Annan and Houghton[242] Divya *et al.*[243] Khovidhunkit *et al.*[244] Dodehe *et al.*[214] Dash and Murthy[113] Nirmala and Karthiyayini[245] Akkol *et al.*[246] Ghosh *et al.*[247] Fikru *et al.*[248] Ruszymah *et al.*[249]

Phytochemical class	Phytochemicals	Plant	Bioactivity	Reference
Phenolics	Tannins	*Phyllanthus muellerianus, Terminalia arjuna, Terminalia avicennioides, Terminalia bellirica, Terminalia chebula and Terminalia coriacea*	• Antimicrobial • Antioxidant properties	Choudhary[250] Agyare *et al.*[251] Isaiah[252] Choudhary[253] Khan *et al.*[254]
	Substituted cinnamic acids: chlorogenic acid, caffeic acid and ferulic acid	*Buddleja globosa, Scorzonera cana var. jacquiniana and S. eriophora and Angelica sinensis.*	• Free radical scavengers	Mensah *et al.*[40] Majewska and Gendaszewska-Darmach[255] Sutar *et al.*[222]
	Phenolic acids such as gallic acid	*Ageratum conyzoides, Emblica officinalis, Punica granatum, Salvia hypoleuca, Schinus lentiscifolius, Strobilanthes crispus, Quercus infectoria, Ximenia americana.*	• Free radical scavenger	Oladejo *et al.*[101] Chidambara Murthy *et al.*[159] Umachigi *et al.*[256] Sachin *et al.*[257] Sumitra *et al.*[258] Pirbalouti *et al.*[259] Estakhr and Javdan[260] Al-Henhena *et al.*[261] Le *et al.*[262] Gehrke *et al.*[263]
	Phenyl propanoids such as glycosides	Asteraceae, Labiateae, Liliceae, Oleaceae, *Ajuga reptans* and *Syringa vulgaris*	• Powerful antioxidants • Anti-inflammatory • Wound healing	Korkina *et al.*[264]
Heteropolysaccharides	Arabinogalactans and rhamnogalacturonans	*Alstonia boonei, Biophytum petersianum Klotzch, Cochlospermum tinctorium Perr, Glinus oppositifolius, Opuntia ficus-indica, and Parquetina nigrescens.*	• Accelerate re-epithelialization and remodeling • Immunomodulatory action • Stimulate cell proliferation	Galati *et al.*[265] Nergard *et al.*[266] Inngjerdingen *et al.*[267] Trombetta *et al.*[120] Inngjerdingen *et al.*[268] Agyare *et al.*[269]

1.7.4. *Terminalia sericea* investigated in this study

Figure 8: *Terminalia sericea* Burch. ex DC.[200]

Terminalia sericea Burch. ex DC. belongs to the family Combretaceae (Figure 8). It is found in the sandy savannah areas in the northern parts of South Africa and in areas of Namibia, Botswana, Zimbabwe, Angola, Mozambique, Zambia, Malawi and Tanzania in Africa.[70] *T. sericea* is indigenous to Africa and has received attention for its traditional use over the years.[152] With regards to wound healing, the dried leaves are powdered and directly applied to wounds to stop bleeding. Additionally it is used as an antibiotic for infected wounds and burns or mixed with cold water to wash a burned area.[270-272] Other reported traditional uses include drinking infusions made from boiling the leaves and roots for the treatment of colds, coughs, fevers, diarrhoea and stomach ailments.[270,271,273-275] Traditional uses of various other parts of the plant are used for the treatment of pneumonia, diabetes, tuberculosis, gonorrhoea, dysentery, and menorrhagia.[270, 271,273-277]

1.8. Aim and objectives

1.8.1. Study aim

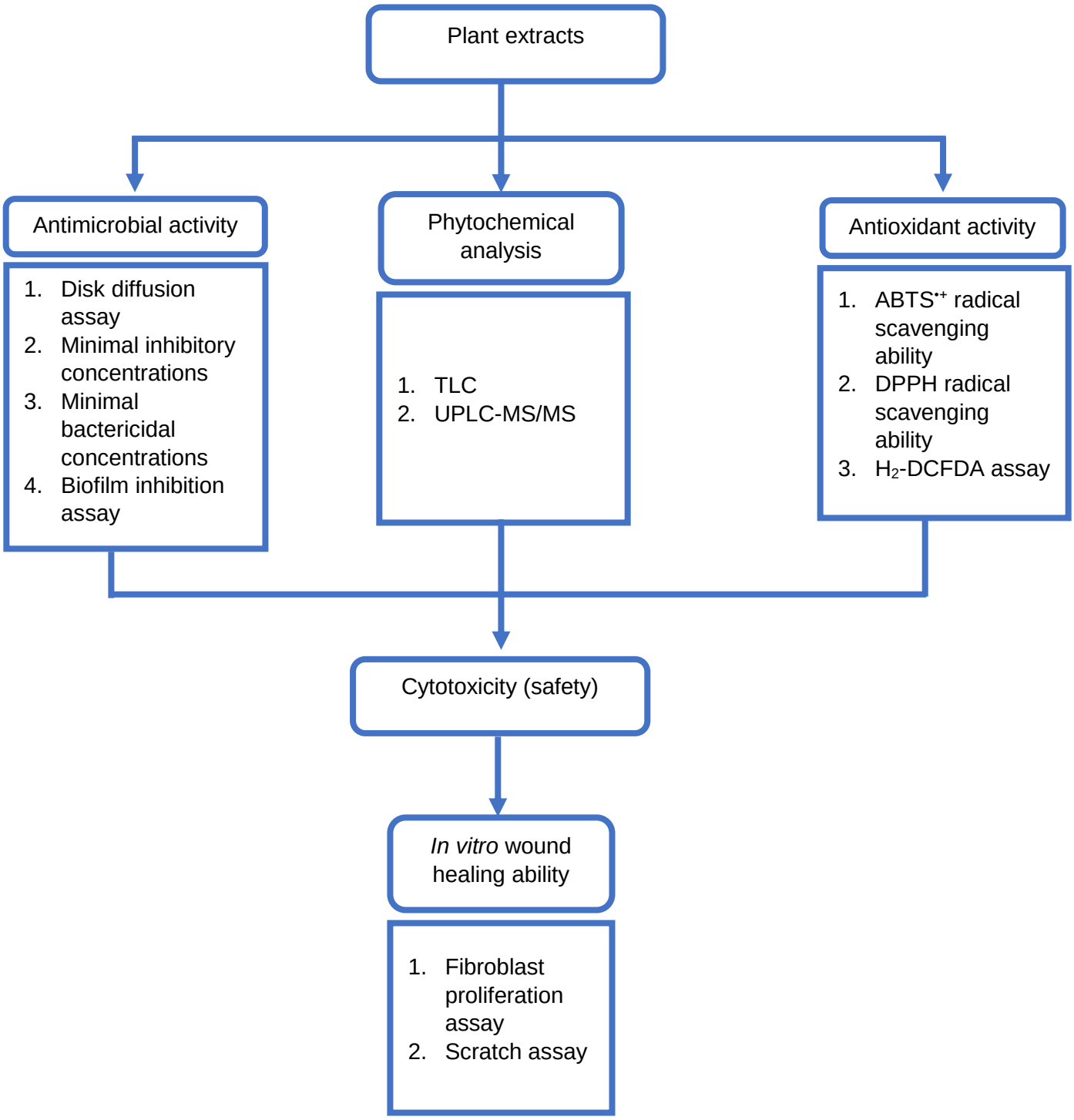
The aim of this study was to assess the wound healing potential of *Terminalia sericea.*

1.8.2. Objectives

The objectives of this study were to:

- determine the phytochemical profile of the crude extracts using thin layer chromatography and UPLC-MS/MS.
- determine the antibacterial activity of the crude extracts against various strains of Gram-positive and Gram-negative bacteria using the disk diffusion and micro-well dilution methods.
- determine the biofilm inhibitory activity of crude extracts using the Crystal violet assay.
- determine the antioxidant potential of *T. sericea* crude extracts using the $ABTS^{\bullet+}$ and DPPH radical scavenging ability assays and to evaluate the protective effects of the extracts against oxidant induced damage using a cellular antioxidant activity assay.
- evaluate the cytotoxicity of *T. sericea* crude extracts in SC-1 fibroblast and EA.hy926 endothelial hybrid cell lines using the sulphorhodamine B assay.
- assess the *in vitro* wound healing potential of *T. sericea* extracts using the fibroblast proliferation and scratch assays.

1.8.3. Project outline



Chapter 2: Materials and methods

Ethics approval to carry out the study was obtained from the Ethics Committee of the Faculty of Health Sciences at the University of Pretoria (Appendix I). A list of reagents and solutions used in this study are provided in Appendix II.

2.1. Plant material

Terminalia sericea roots and leaves were collected and the identity was confirmed by Dr N Hahn (Biodiversity research chair, University of Venda). A voucher specimen (N.H. 1878) is deposited at the Soutpansbergenesis herbarium, Machado. The plant material was air-dried, ground to a fine powder and stored in amber bottles, until used.

2.2. Extract preparation

Ethnomedicinal herbal remedies were prepared as infusions or decoctions using hot water whereas pharmaceutical agents were prepared through maceration or percolation with organic solvents. Four crude extracts of *T. sericea* were prepared using different solvents of varying polarity. Aqueous extracts were prepared using hot water crude extraction, whereas, methanol, ethyl acetate and hexane extracts were prepared using maceration.

For the hot water crude extraction, 30 g of the leaf and root mixture was added to 300 mL boiling water which was stirred for 15 min to simulate tea brewing. The mixture was centrifuged for 5 min at 1000 *g*, filtered through a 0.2 µm filter and concentrated through *in vacuo* lyophillization (Freezone® 6 Freeze Dry System, Labconco, Canada). For the methanol, ethyl acetate and hexane maceration, 30 g of plant material was added to 300 mL of the respective solvent and sonicated for 30 min, shaken for 2 h and incubated at 4˚C for 16 h. This procedure was repeated three times by replenishing the solvent and with shaking. Extracts were centrifuged for 5 min at 1000 *g,* filtered through a 0.2 µm filter and concentrated through *in vacuo* rotary evaporation (Büchi Rotovapor R-200, Büchi). Dried crystals were reconstituted in distilled water and lyophilized. Yields were determined gravimetrically. All extracts were reconstituted in either phosphate buffered saline (PBS) or dimethyl sulfoxide (DMSO) and diluted to a concentration of 100 mg/mL, which was aliquoted and stored at -80˚C until use.

2.3. Phytochemical analysis

2.3.1. Thin layer chromatography

Phytochemicals are the bioactive components in plants that are responsible for its medicinal properties. In recent years, identification of phytochemicals using chromatography has assumed a pyramid strategy, where thin layer chromatography (TLC) or high performance thin layer chromatography (HPTLC) serves as screening step to identify broad classes of phytochemicals present in a plant.[278-281] The screening step is followed by a confirmation step which then narrows down specific phytochemicals within these classes. This is done by employing quantitative analytical chromatographic techniques such as high performance liquid chromatography (HPLC), matrix-assisted laser desorption/ionization (MALDI), ultra performance liquid chromatography (UPLC) and mass spectrometry (MS).[278,279,281-283]

The absence or presence of major phytochemical classes in the crude extracts was determined using thin layer chromatography following the method of Howard *et al.*[280] and EMD chemicals.[284] One microliter of each crude extract (10 mg/mL) was spotted onto a 10 cm × 10 cm aluminium silicate plate (Merck, Darmstadt, Germany) and allowed to dry under a fume hood. The plate was placed in a saturated glass tank containing the relevant mobile phase for the phytochemical class being investigated (Table 5). Following development, plates were allowed to dry and visualised under a UV lamp at 366 and 254 nm in order to detect compounds with native fluorescence. The presence and colour of the spots under UV light was recorded. The plates were then sprayed with the appropriate spray reagent for the phytochemical class investigated (Table 4) and incubated at 40ºC for 5 min.

Table 5: Mobile phases and spray reagents utilised to detect phytochemical classes.

Phytochemical	Mobile phase	Spray reagent	Reference
Alkaloids	Chloroform:Acetone:Methanol 4:4:2	Dragendorff	Howard *et al.*[280]
Coumarins	Ethyl acetate:Chloroform 60:40	5% Methanolic potassium hydroxide	Howard *et al.*[280]
Flavonoids	Chloroform:Acetone:Methanol 4:4:2	3% Sodium nitrate:1% aluminum trichloride:0.5M sodium hydroxide	Howard *et al.*[280]
Glycosides	Ethyl acetate:Methanol:ddH_2O 100:13.5:10	Trichloroacetic acid in chloroform and hydrogen peroxide	Howard *et al.*[280]
Phenolic acids	Methanol:Ammonium hydroxide 200:3	Folin-Ciocalteau	Howard *et al.*[280]
Saponins	Chloroform:Methanol:Water 60:35:5	1% Vanillin 5% sulphuric acid	EMD chemicals[284]
Sterols	Chloroform:Methanol 3:4	85% Phosphoric acid in ddH_2O	Howard *et al.*[280]
Terpenoids	Toluene:Ethyl acetate 93:7	1% Vanillin 5% sulphuric acid	EMD chemicals[284]

2.3.2. UPLC-TOF-MS

2.3.2.1. UPLC analysis

A Waters UPLC coupled in tandem to a Waters photodiode array (PDA) detector and a SYNAPT G1 HDMS mass spectrometer was used to generate accurate mass data. Optimisation of the chromatographic separation was done utilising a Waters HSS T3 C18 column (150 mm x 2.1 mm, 1.7 µm) which was maintained at 60°C. A binary solvent mixture was used consisting of water (Eluent A) containing 10 mM formic acid (natural pH of 2.3) and acetonitrile (Eluent B) containing 10 mM formic acid. The initial conditions were 95% A at a flow rate of 0.4 mL/min which was maintained for 1 min, followed by a linear gradient to 5% 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

was 1 - 3 µL. The PDA detector was scanned between 200 and 500 nm (1.2 nm resolution) while collecting 20 spectra per second.

2.3.2.2. TOF mass spectrometry analysis

The SYNAPT G1 mass spectrometer was used in V-optics and operated in electrospray mode to enable detection of phenolic and other ESI-compatible compounds. Leucine enkephalin (50 pg/mL) was used as reference calibrant to obtain typical mass accuracies between 1 and 5 mDalton (mDa). The mass spectrometer was operated in ESI positive and negative mode with a capillary voltage of 2.5 kV, the sampling cone at 30 V and the extraction cone at 4.5 V. The scan time was 0.1 seconds covering the 100 to 1400 Dalton mass range. The source temperature was 120°C and the desolvation temperature was set at 450°C. Nitrogen gas was used as the nebulisation 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 systems well as for data manipulation.

A pure standard of punicalgin (Sigma-Aldrich, South Africa) was run through the UPLC-TOF-MS system and a fingerprint of the compound was obtained. Punicalagin eluted as a split peak (isomers) of mass 541.0081 m/z and 541.0112 m/z respectively in electro spray ionisation (ESI) negative mode at retention times 3.11 and 3.45 min respectively (Appendix IV A-D). The masses of the two peaks added up the mass of punicalagin (1084.72 m/z). The monoisotopic masses and chemical structures for sericoside (666.398 m/z), anolignan B (266.131 m/z) and arjunic acid (488.350 m/z) were obtained from literature and a targeted analysis was performed on the chromatogram fingerprint (base peak) of each extract of *T. sericea* in order to identify the respective compounds.

2.4. Antioxidant activity

2.4.1. $ABTS^{\bullet+}$ decolourisation assay

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$) assay is a decolorization assay used for both hydrophilic and lipophilic antioxidants, where spectrophotometry is used to measure the reduction of $ATBS^{\bullet+}$ to ABTS which results in the loss of colour from blue/green to lighter shades.[285,286] The $ABTS^{\bullet+}$ decolourisation assay was used to assess $ABTS^{\bullet+}$ radical scavenging ability following the method of Re *et al.*[47]

A stock solution of 7.46 mM $ABTS^{\bullet+}$ salt (Sigma-Aldrich, South Africa) and 2.5 mM potassium persulfate (Sigma-Aldrich, South Africa) was prepared in distilled water and allowed to stand in the dark for 16 h at 4°C. After incubation, the $ABTS^{\bullet+}$ solution was diluted with distilled water to an absorbance of 0.70 ± 0.02 at 734 nm using a spectrophotometer (Lambda UV/VIS Spectrophotometer, Perkin Elmer). Into a 96-well plate, was added 20 µL of either; distilled water (negative control), crude extracts (half log dilutions 0.1, 0.3, 1.0, 3.2, 10.0 and 32.0 µg/mL) or 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), (half log dilutions 0.1, 0.3, 1.0, 3.2, 10.0 and 32.0 µg/mL; positive control). This was followed by the addition of 180 µL of the $ABTS^{\bullet+}$ solution. A colour control was included for the highest concentration of each extract to ensure that no quenching occurred (20 µL of crude extracts followed by 180 µL of distilled water). The absorbance readings were recorded at 405 nm (Synergy 2, Bio-Tek Instruments, Inc.). The $ABTS^{\bullet+}$ scavenging capacity of the extract was compared with that of Trolox and the percentage inhibition calculated as percentage of the negative control:

$$ABTS^{\bullet+}\ radical\ scavenging\ ability = \left[\frac{Asample}{Acontrol}\right] \times 100$$

Where:

Acontrol is the absorbance of $ABTS^{\bullet+}$ radical

Asample is the absorbance of $ABTS^{\bullet+}$ radical + sample extract/standard.

2.4.2. DPPH radical scavenging assay

The 2.2-diphenylpicrylhydrazyl (DPPH) assay is based on the ability of the antioxidant being tested to stabilise DPPH, a free radical, by donating a hydrogen atom, which results in the bleaching of a deep violet to lighter shades which can be measured spectrophotometrically.[285] The DPPH radical scavenging ability of the extracts was measured as described by Gyamfi *et al.*[287] with minor modifications. A solution of 240 µM DPPH in methanol was prepared and sonicated for 20 min. The DPPH solution (180 µL) and 20 µL of either; varying concentrations of the plant extracts (half-log dilutions 0.1, 0.3, 1, 3.2, 10.0 and 32.0 µg/mL), distilled water (negative control) or Trolox (0.1, 0.3, 1, 3.2, 10.0 and 32.0 µg/mL; positive control) was added to the wells of a 96-well plate. The plates were incubated for 15 min in the dark at room temperature. The absorbance was measured at 570 nm using a microplate reader (Synergy 2, Bio-Tek

Instruments, Inc.). Trolox was used as a reference antioxidant compound. The ability of the plant extracts to scavenge the DPPH radical was calculated as a percentage of the negative control using the equation:

$$DPPH\ radical\ scavenging\ ability\ (\%) = \left[\frac{Asample}{Acontrol}\right] \times 100$$

Where:

Acontrol is the absorbance of DPPH radical + methanol

Asample is the absorbance of DPPH radical + sample extract/standard.

2.4.3. Cellular antioxidant activity assay

The cellular antioxidant activity (CAA) assay measures the protective antioxidant activity that a test compound provides to cells when challenged with a free radical or oxidant. This assay measures the inhibition exerted by an antioxidant test compound on the oxidation of an intracellular probe, 2', 7'-dichlorodihydrofluorescein diacetate (H_2-DCFDA) which diffuses into cells, where it is hydrolysed into DCFDA. The peroxyl radicals generated from an oxidant such as, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), oxidise the DCFDA to the fluorescent derivative DCF.[288] When antioxidants are added to the system, quenching of the peroxyl radicals occur and the production of DCF is inhibited. The rate of increase in cellular fluorescence as compared to control cells indicates the cellular antioxidant activity of the test compound.[288]

The cellular antioxidant activity of the crude extracts was measured following the method as described by Blasa *et al.*[288] with minor modifications. SC-1 fibroblast cells were maintained and prepared as described in section 2.6.1 (page 53). A volume of 100 µL of cells (5 x 10^4 cells/mL) were seeded into the wells of a white 96-well plate (Cellstar). The plates were incubated for 24 h at 37ºC and 5% CO_2 to allow for attachment of the cells. A volume of 100 µL (10 µM) of H_2-DCFDA, was added to each well and the plate was incubated in the dark at 37ºC at 5% CO_2 for 30 to 60 min. Following the incubation period, plates were washed twice with PBS using a multichannel pipette. Half-log dilutions of the crude extracts (0.1 - 32 µg/mL) were prepared in PBS and 100 µL added to respective test wells. To the control wells, 100 µL of either Trolox (antioxidant positive control), PBS (negative control), AAPH (oxidant positive control) or DMSO (vehicle control) was added and the plate was allowed to

incubate for 1 h in the dark. Following pre-treatment, 50 µL of APPH (5 mM) was added to each well with the exception of the negative control wells. The plate was placed into a fluorometer (Synergy 2, Bio-Tek Instruments, Inc.) and the fluorescence read at an excitation wavelength of 485 nm and emission wavelength of 520 nm every minute for 4 h. The area under the curve was determined using GraphPad prism 5.0 and the percentage of intracellular ROS was calculated as compared to the negative control.

2.5. Antibacterial activity

2.5.1. Microorganisms

The antibacterial activity of the crude extracts was assessed against standard Gram-positive and Gram-negative bacteria obtained from the American Type Culture Collection (ATCC). Gram-positive bacteria included: *Bacillus cereus* (ATCC 10876), *Staphylococcus aureus* (ATCC 33591), *Enterococcus faecalis* (ATCC 49532), and *Staphylococcus epidermidis* (ATCC 13518). Gram-negative bacteria included: *Vibrio parahaemolyticus* (ATCC 17802), *Pseudomonas aeruginosa* (ATCC 19429), *Proteus vulgaris* (ATCC 6380), *Shigella flexineri* (ATCC 12022) and *Escherichia coli* (ATCC 35218).

2.5.2. Disk diffusion assay

The disk diffusion assay was performed according to CLSI guidelines.[289] Bacteria were maintained in Mueller-Hinton broth. Fresh 24 h cultures were grown for the assay, after which a stock inoculum of each test microbe was prepared and adjusted to a 0.5 McFarland's standard (equivalent to a 5×10^5 CFU/mL) using a spectrophotometer (SPEKOL 1500/1 Analytik jena, Germany). A pour plate was created by adding 100 µL of the stock inoculum to approximately 25 mL of Mueller-Hinton agar at 37ºC in a McCartney bottle. The bottle was inverted and poured into 90 mm × 90 mm agar plate and allowed to set. Two pour plates were created for each microbe, one containing the four plant extracts and the other containing the vehicle control (DMSO), the respective positive control (Table 6) and the negative control (blank disk).

Whatmann disks (6 mm) (Sigma-Aldrich, South Africa) were impregnated with 12 µL of the respective *T. sericea* crude extract (300 µg/mL for MeOH, HW and EtOAc, and 12 µg/mL for HE) and allowed to dry for 5 min. The dried disks were placed on the set agar surface and the plate was incubated in an inverted position at 37°C for 24 h. The zone

of inhibition was determined by measuring the clear area at 3 points using a digital calliper. Studies were performed in triplicate and results expressed as means along with the standard deviation (SD) of three parallel measurements.

Table 6: Antibacterial controls used in antimicrobial assays.

Microorganism	Antibiotic (control)	Concentration		
		Disk diffusion (µg)	MIC (µg/mL)	Biofilm (µg/mL)
Gram- positive				
Bacillus cereus	Gentamicin	10	0.391 – 100	1.56 – 100
Enterococcus faecalis	Ampicillin	10	0.391 – 100	1.56 – 100
Staphylococcus aureus	Ciprofloxacin	5	0.391 – 100	1.56 – 100
Staphylococcus epidermidis	Ciprofloxacin	5	0.391 – 100	1.56 – 100
Gram- negative				
Escherichia coli	Ciprofloxacin	5	0.391 - 100	1.56 - 100
Proteus vulgaris	Ciprofloxocin	5	0.391 - 100	1.56 - 100
Pseudomonas aeruginosa	Ciprofloxacin	5	0.391 - 100	1.56 - 100
Shigella flexineri	Ampicillin	10	0.391 - 100	1.56 - 100
Vibrio parahaemolyticus	Ciprofloxocin	5	0.391 - 100	1.56 - 100

2.5.3. Broth microdilution assay

Minimum inhibitory concentrations (MIC's) were determined against microorganisms in which activity (cut-off) was noted during the disk diffusion assay. This was done using the broth micro-dilution assay described by Eloff.[45] A working inoculum of each microorganism (24 h cultures) was prepared and adjusted to 0.5 McFarland standard turbidity. Serial dilutions of the crude extracts were prepared in Mueller-Hinton broth giving rise to a range of ten concentrations (0.01 – 25 mg/mL). A 96-well plate was prepared and 100 µL of the working inoculum was added to each well, with the exception of the blank wells to which 100 µL of broth was added. To test wells, 100 µL of either, the plant extract, a positive control (Table 6), a negative control (broth) or a vehicle control (DMSO) was added. The microplate was then incubated at 37°C for 24 h. After

incubation, 20 µL of *p*-iodonitrotetrazolium (INT) at a concentration of 3 mg/mL was added to each well and incubated for 2 h. Results were analysed visually. The MIC of the positive control and the sample were defined as the lowest concentration of the sample that was able to visibly inhibit the growth of the bacteria via a colour change, where a colourless solution indicated inhibition of microbial growth and a red/pink solution indicated uninhibited microbial growth.

Minimum bactericidal concentration (MBC) of the plant extracts against test isolates was determined by taking 5 µL of test dilution from each well in the MIC assay, which did not show physical growth, and sub-culturing it on nutrient agar plates. Plates were incubated for a further 24 h at 37°C. The complete absence of growth at the applied concentration was considered as the MBC.

2.5.4. Biofilm formation

Biofilms were grown under sterile conditions in 96-well micro-plates as described by O'Toole (2011)[28] and Sarkar *et al.* (2014).[29] Fresh 24 h microbial cultures were diluted to 1 x 10^5 CFU/mL with Mueller-Hinton broth and 50 µL was transferred to each of the test wells with the exception of the blank wells to which 50 µL of broth was added. The plate was allowed to incubate at 37°C for 24 h.

2.5.5. Biofilm inhibition assay

Biofilms were grown as described in 2.5.4. Only the crude extracts and microorganisms which showed activity after MIC determination were tested. Serial dilutions (0.039, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5 and 5 mg/mL) of the MeOH, HW and EtOAc crude extracts and HE extract (0.007, 0.016, 0.031, 0.063, 0.125, 0.25 and 1 mg/mL) were prepared in Mueller-Hinton broth. A volume of 50 µL of each concentration was transferred to the test wells of microbial cultures in the micro-plate. Serial dilutions of the respective antimicrobial drugs listed in Table 5 were made in the same manner as the crude extracts and served as positive controls. The plate was allowed to incubate for a further 24 h at 37°C.

2.5.6. Crystal Violet assay

The Crystal Violet assay was performed according to the method developed by O'Toole[28] and Sarkar *et al*.[29] Following incubation the micro-plate was emptied and rinsed with distilled water using a multichannel pipette, to remove loosely

attached/planktonic bacteria, after which, the plates were left to dry for 45 min. A volume of 200 µL of 0.1% Crystal Violet was added to each well of the micro-plate which was left to stain for 30 min at room temperature. The plates were then rinsed three times with distilled water to remove unabsorbed stain and left to dry for 24 h. Ethanol (95%; 200 µL) was added to the wells, to solubilise the stained biofilms, and incubated for 15 min. The plates were read at 560 nm using a micro-plate reader (GLR 1000, Genelabs Diagnostics, South Africa).

2.6. *In vitro* cytotoxcity

2.6.1. Cell lines

Cell cultures were purchased from the American Type Culture Collection (ATCC). The EA.hy926 (ATCC-CRL-2922), endothelial hybrid, cell line and the SC-1 (ATCC- CRL-1404) mouse fibroblast cell line were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) with 1% penicillin/streptomycin. Cells were incubated in a humidified incubator at 37^0C with 5% CO_2 until confluent.

2.6.2. Preparation of cells

Cells were harvested through trypsinization and centrifuged at 200 *g* for 5 min. Seeding density was determined using the Trypan Blue exclusion method and a haemocytometer. The cells were then diluted to a concentration of 2 x 10^5 cells/mL for the EA.hy926 cell line and 5 x 10^4 cells/mL for SC-1 cell line using DMEM supplemented with 10% FCS.

2.6.3. Suphorhodamine B assay

The Sulphorhodamine B (SRB) assay is based on the ability of the sulfonic groups contained within the SRB dye to bind to protein components (such as amino acid residues) in fixed cells. The dye binds to these protein components under acidic conditions and dissociates under basic conditions. As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell mass.[290]

The cytotoxicity of the crude extracts was determined using the SRB assay as described by Vichai and Kirtikara[290], with minor modifications. A volume of 100 µL of cells were seeded into a 96-well plate at a concentration of 2 x 10^4 cells/well and incubated for 24

h to allow cells to attach to wells. Blank wells contained 200 µL 5% FCS-supplemented DMEM. Cells were exposed to 100 µL non-supplemented DMEM (negative control), 0.5% DMSO (vehicle control), 1% saponin (positive control) or crude extracts (2, 6.4, 20, 64, 200 µg/mL in DMEM) for 24 h or 72 h. After incubation, cells were fixed with 100 µL cold 50% trichloroacetic acid. The plate was then incubated for 24 h at 4°C, after which it was washed four times with slow-running tap water. Plates were allowed to dry at 40°C. The SRB (0.057%; 100 µL) was added to each well and allowed to stain for 30 min. Excess dye was removed by washing four times with 100 µL acetic acid (1%) where after plates were allowed to dry at 40°C. Bound SRB was solubilized by the addition of 200 µL of trisaminomethane (Tris) base solution (10 mM, pH 10.5) and plates shaken for 30 min. Absorbance was measured at 510 nm (reference 630 nm) using a microplate reader (Synergy 2, Bio-Tek Instruments, Inc.) and the percentage viability determined relative to the negative control.

2.7. Cell proliferation and migration

2.7.1. Fibroblast proliferation assay

Fibroblast proliferation was assessed using the method as described by Steenkamp *et al.*[291], with minor modifications. SC-1 cells (100 µL) were seeded into a 96-well plate at a density of 2.5 × 10^5 cells per well and incubated for 24 h. Thereafter, the cells were exposed to 100 µL of either, half-log dilutions of the extracts (0.02- 64 µg/mL), 15% FCS (positive control), DMEM (negative control) or 0.5% DMSO (vehicle control) and incubated for 24 h. The proliferation was assessed using the SRB assay as described in section 2.6.3 (page 53). Absorbance was measured at 510 nm (reference 630 nm) using a microplate reader (Synergy 2, Bio-Tek Instruments, Inc.) and the percentage viability was determined relative to the negative control.

2.7.2. Scratch assay

The wound scratch assay measures the expansion of a cell population on surfaces. The spreading and migration capabilities of the SC-1 and EA.hy926 cells were assessed using the wound scratch assay of Fronza *et al.*[73] as modified by Hostanska *et al.*[292] Cells were seeded (800 µL) into a 24-well tissue culture plate at a concentration of 1.75 ×10^5 cells per well and cultured in DMEM supplemented with 10% FCS and incubated at 37ºC and 5% CO_2 for 24 h to nearly confluent cell monolayers. The medium was aspirated from wells using a pipette and replaced with DMEM supplemented with 0.5%

FCS and incubated for a further 24 h. Thereafter two linear wounds were generated in the monolayer using a sterile 100 µL plastic pipette tip. The first scratch was created vertically and the second intersecting the vertical scratch horizontally (Figure 9). The intersection was then used to maintain reference points. Any cellular debris was removed by washing the cells once with PBS. The scratch was measured using a phase contrast microscope coupled to a real time camera (Zeiss inverted Axiovert CFL40). A baseline image was captured for each well. The cells were then treated with different concentrations of the plant extracts (0.3, 1, 3.2, 10 and 32 µg/mL), platelet derived growth factor (PDGF) (positive control), 0.5% DMSO (vehicle control) and a negative control (untreated) and incubated for 24 h. The plates were re-examined microscopically after the incubation period and photographic images were taken. The images were further analysed using computing software (Axiovision 3.0 and Image J) to determine the width of the 'wound'. Wound closure was evaluated using the following formula:

$$Wound\ closure\ \% = \left[\frac{test\ compound\ \% - untreated\ control\ \%}{confluent\ area\ \% - untreated\ control\ \%}\right] \times 100$$

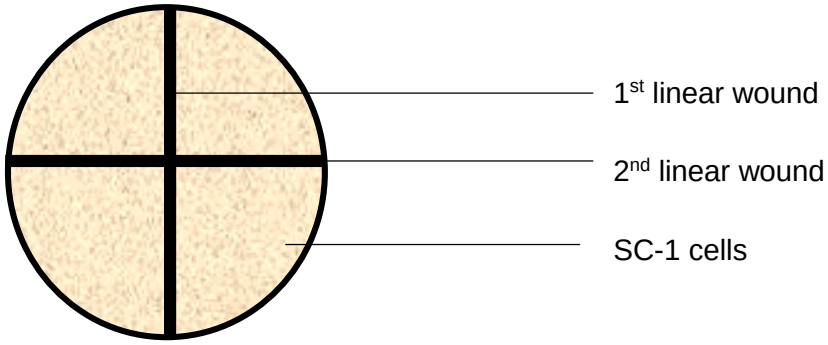


Figure 9: An illustration of wound generation in a well for conducting the scratch assay.

2.8. Statistics

All experiments were performed in triplicate on three separate occasions. Statistical analysis was performed using GraphPad Prism 5.0. Significance was taken at $p < 0.05$. The statistical tests for each assay is described individually.

$ABTS^{\bullet+}$ radical scavenging ability: The percentage inhibition of $ABTS^{\bullet+}$ was determined relative to the negative control. A half-maximal inhibitory concentration (IC_{50}) value was

calculated from a standard curve generated through non-linear regression (normalized variable slope) indicating the relationship between extract concentration and $ABTS^{\bullet+}$ radical scavenging ability. One-way ANOVA was performed to analyse the differences in the means between the crude extracts and Trolox (positive control).

DPPH radical scavenging ability: The percentage inhibition of DPPH was determined relative to the negative control. An IC_{50} value was calculated from a standard curve generated through non-linear regression (normalized variable slope) indicating the relationship between extract concentration and DPPH radical scavenging ability. One-way ANOVA was performed to analyse the differences in the means between the crude extracts and Trolox (positive control).

Cellular antioxidant activity assay: The area under the curve (AUC) was calculated using GraphPad prism 5.0 and the percentage of intracellular ROS was calculated as compared to the negative control. One-way ANOVA was performed to analyse the differences in the means between the crude extracts and Trolox (positive control) and the negative control.

Disk diffusion assay: The zone of inhibition was measured using digital callipers. The average of three measurements were used and results were reported in mm ± SD. Only complete zones of inhibition were taken into account.

Broth microdilution assay: MIC's were determined visually. The MIC is the lowest concentration which inhibits microbial growth. Uninhibited microbial growth was indicated by a pink/red colour, whereas inhibition of microbial growth was indicated by failure of well to change colour.

Minimum bactericidal concentration: The MBC was taken as the lowest concentration which showed no colony forming units or microbial growth on agar plates on all occasions.

SRB assay: Cell density was assessed by calculating the IC_{50} value from the generated data. The relationship between the extract concentration and cell density was determined from the standard curve generated through non-linear regression (normalized variable slope).

Scratch assay: Wound closure was expressed as a percentage relative to the negative control. Results are reported as mean percentage ± the standard deviation (SD).

Fibroblast proliferation assay: Cell density was assessed by calculating the IC_{50} value from the generated data on GraphPad prism 5.0. The relationship between the extract concentration and cell viability was determined from the standard curve generated through non-linear regression (normalized variable slope).

Chapter 3: Results and discussion

3.1. Phytochemical analysis

Four solvent extracts were prepared and the yields determined gravimetrically. The methanol extract produced the highest yield (Table 7). Due to its non-polar nature, the hexane extract did not dry completely with freeze-drying and produced a sticky yellow paste that was reconstituted in DMSO.

Table 7: Extraction yield and appearance of the crude extracts.

Solvent	Extraction yield (%)	Appearance
Methanol (MeOH)	9.17	Light brown powder
Hot water (HW)	2.98	Dark brown powder
Ethyl acetate (EtOAc)	3.29	Yellow powder
Hexane (HE)	0.083	Sticky yellow paste

The phytochemical classes detected in the crude extracts are provided in Table 8. Alkaloids, coumarins, flavonoids, glycosides, phenolic acids, saponins, sterols and terpenoids have been identified in *T. sericea* in different studies[293,294], supporting the results obtained. The Rf values corresponded to the presence of the specific phytochemical class (Table 8 and Appendix III). Furthermore, UPLC-TOF-MS confirmed the presence of phytochemicals characteristic of *Terminalia* species: punicalagin, sericoside, anoligan B and arjunic acid (Table 9 and Appendix IV).

Alkaloids were present in the MeOH, EtOAc and HE crude extracts (Table 8). These basic nitrogenous compounds occur most abundantly in the plant kingdom.[295] Alkaloids have been detected in the leaf extracts of *T. sericea*, *T. coriacea* and *T. catappa.* [293,294,296-298]

Coumarins are a class of lactones characterised by a benzene-ring fused to α-pyrone-ring, which possesses a conjugated system with rich electron and good charge-transport properties.[299,300] Coumarins were detected in the MeOH, EtOAc and HE extracts of *T. sericea* (Table 8). Coumarins have been reported to be present in the leaves of *T. sericea.*[294] Other *Terminalia* spp. found to contain coumarins include *T. spinosa* (methanolic leaf extracts)[301], *T. tropophylla* (6,7,8-trisubstituted coumarin)[301]

and *T. catappa* (dichloromethane and ethyl acetate leaf extracts)[302]. Coumarins have been reported to be absent in water extracts, which supports the current findings.[302]

Table 8: Major phytochemical classes detected in the crude extracts.

Phytochemical	Plant extract			
	MeOH	**HW**	**EtOAc**	**HE**
Alkaloids	Yes	No	Yes	Yes
Coumarins	Yes	No	Yes	Yes
Flavonoids	Yes	Yes	Yes	Yes
Glycosides	Yes	No	Yes	Yes
Phenolic acids	Yes	Yes	Yes	Yes
Saponins	Yes	Yes	Yes	Yes
Sterols	Yes	Yes	Yes	Yes
Terpenoids	Yes	No	Yes	Yes

MeOH: methanol; HW: hot water; EtOAc: ethyl acetate; HE: hexane

Flavonoids, identified in all the crude extracts (Table 8), are oxygen-containing aromatic compounds.[303] Flavonoids have been reported in the root extracts[304], acetone stem-bark extracts[305], and methanol and ethyl acetate root extracts[306] of *T. sericea*. Other *Terminalia* spp. containing flavonoids include: *T. coriacea*[296] and *T. arjuna* (stem-bark extracts).[296,307,308] In the latter species, the flavonoids arjunolone, bicalein, kampferol, pelorgonidin, quercetin and luteolin were identified.

Glycosides are conjugates of sugars with small organic molecules which can be classified by their glycone groups, types of glycosidic bonds as well as aglycone groups.[303] Glycosides were identified in the MeOH, EtOAc and HE crude extracts (Table 8). Ellagic acid-glycosides, as well as triterpenoidal glycosides, have been isolated from various *Terminalia* spp.[309] Stilbene glycosides, such as (3'5'-dihydroxy-4-(2-hydroxy-ethoxy) resveratrol-3-O-β-rutinoside, resveratrol-3-β-rutinoside glycoside and 3',4,5'-trihydroxystilbene (resveratrol), have been isolated from ethanol root-bark extracts of *T. sericea.*[310,311]

Phenolic acids were found to be present in all four solvent extracts (Table 8). Phenolic acids are classified into hydroxybenzoic acid derivatives (gallic, ellagic, vanillic and

syringic acids) and hydroxycinnamic acid-derivatives (*p*-coumaric, ferrulic, caffeic, and synaptic acids). Phenolic acids have been identified in extracts of *T. sericea*[306] as well as in other *Terminalia* spp. Phenolic acids identified in the *Terminalia* genus include caffeic acid, ferulic acid, vanillic acid, coumaric acid, *p*-hydroxybenzoic acid and 3,4-dihydroxybenzoic.[309]

Saponins were detected in all of the crude extracts (Table 8). Saponins are a diverse group of naturally occurring compounds which are widely distributed in the plant kingdom, and are characterized chemically by a triterpene or steroid aglycone and one or more sugar chains.[312] Sericoside (Figure 10B) is a triterpenoidal saponin and marker compound of the *Terminalia* genus. The ethanolic root extract of *T. tropophylla* has been reported to contain an oleanane-type triterpenoid saponin, arjunglucoside I and sericoside.[313] The presence of sericoside was confirmed in the HW and EtOAc extracts using UPLC-TOF-MS (Appendix IV E and F). Sericoside was first isolated from the roots of *T. sericea* by Bombardelli and colleagues in 1974.[314] It has also been isolated from *T. tropophylla*, *T. macroptera* and *T. ivorensis.*[309]

Sterols are a vital component of the membranes of all eukaryotic organisms which are produced through *de novo* synthesis or obtained from the environment.[315] Plant sterols appear to control membrane fluidity and permeability as well as signal transduction.[315] Sterols were present in all the crude extracts (Table 8). β-sitosterol and β-stigmasterol have been detected in *T. sericea.*[310]

Terpenoids were present in the MeOH, EtOAc and HE crude extracts. These compounds exist in nature in the form of monocyclic and multi-cyclic structures and are thought to elicit their effects through synergism with other compounds.[199] Arjunic acid (Figure 10D) was identified in the MeOH and EtOAc crude extracts (Table 9 and Appendix IV I and J). Arjunic acid has previously been identified in the ethyl acetate extract of *T. sericea* roots[271], as well as in the fruits, roots, stem-bark and bark of *T. macroptera*[309] and the bark extracts of *T. glaucescens.*[316]

Table 9: Compounds identified in the crude extracts of *T. sericea* using UPLC-TOF-MS.

Compound and mass to charge ratio	Extract	Mass detected (m/z)	Retention time (min)	Mode	Intensity
Punicalagin 1084.720 (m/z)	MeOH	541.0256 and 541.0260 (Split-peak)	3.11 and 3.45	Negative	2.77×10^3
	HW	541.0211 and 541.0306 (Split-peak)	3.12 and 3.46	Negative	3.42×10^3
	EtOAc	541.0176 and 541.0165 (Split-peak)	3.11 and 3.46	Negative	2.99×10^3
Sericoside 666.398 (m/z)	HW	689.3724 (Na^{23} adduct)	7.08	Positive	214
	EtOAC	689.3724 (Na^{23} adduct)	7.08	Positive	858
Anolignan B 266.131 (m/z)	MeOH	267.1311	10.10	Positive	136
	EtOAc	267.1293	10.10	Positive	393
Arjunic acid 488.350 (m/z)	MeOH	487.3558	10.00	Negative	416
	EtOAc	487.3447	10.00	Negative	1.78×10^3

MeOH: methanol; HW: hot water; EtOAc: ethyl acetate; HE: hexane

Although tannins and lignans were not assessed using TLC, punicalagin and anolignan B was identified using UPLC-TOF-MS (Table 9). The latter two phytochemicals are markers of the *Terminalia* genus.[296,309] Punicalagin (Figure 10A) was identified in the MeOH, HW and EtOAc extracts of *T. sericea* (Table 9 and Appendix IV A and D). Punicalagin is an ellagitannin containing a gallagyl-moiety which was originally isolated from the fruit peel of *Punica granatum.*[317] As a marker, it is not surprising that punicalagin has been detected in various *Terminalia* spp.; including, *T. chebula*, *T. bellerica*, *T. horrida*, *T. oblongata, T. brachystemma, T. macroptera, T. catappa, T. arjuna and T. myriocarpa*[309] and for the first time, to the best of the authors knowledge, in *T. sericea*.

Anolignan B (Figure 10C), a lignin compound, detected in the MeOH and EtOAc crude extracts (Table 9 and Appendix IV G and H) has previously been isolated from the ethyl acetate extracts of *T. sericea*[318], and has also been identified in the fruit rind extracts of *T. bellirica.*[319]

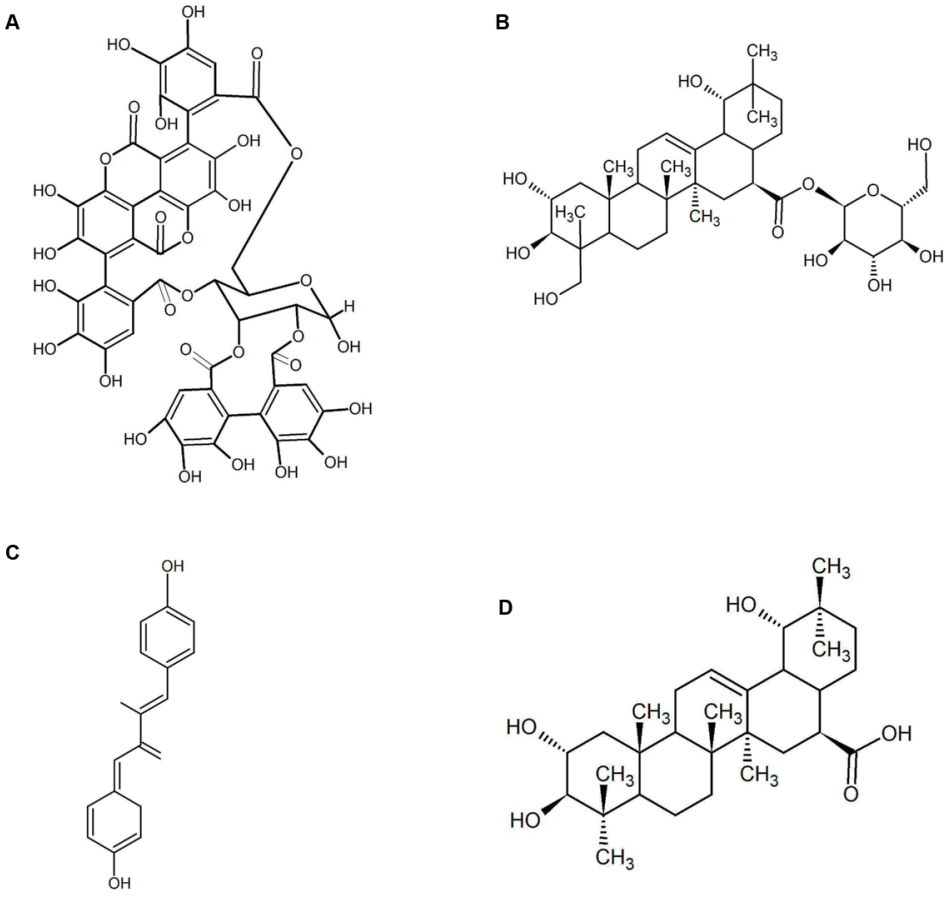


Figure 10: Chemical structure of A) punicalagin[320] B) sericoside[296], C) anolignan B[321] and D) arjunic acid.[296]

3.2. Antioxidant activity

Plants produce an array of antioxidant compounds which have been proven to be highly effective in controlling free radical production, preventing undesirable effects caused by ROS and supporting the organisms' inherent antioxidant and detoxifying mechanisms.[322,323] The mechanisms whereby antioxidants provide protection can be classified into two broad groups, hydrogen-atom transfer (HAT) and single-electron transfer (SET).[324] The HAT mechanism occurs when antioxidant compounds quench free-radical species by donating hydrogen atoms whereas the SET mechanism occurs when an antioxidant transfers a single electron to aid in the reduction of potential target

compounds.[324] It is for this reason that antioxidant activity should not be concluded on the basis of a single antioxidant test model.[285] Various assays have been developed for the detection of antioxidant activity, each examining different mechanisms.[325] The radical scavenging abilities of the crude extracts of *T. sericea* were measured using the CAA assay (HAT mechanism) and $ABTS^{\bullet+}$ and DPPH (SET mechanism) assays. Furthermore, antioxidant activity was confirmed in a cellular model of oxidation.

The $ABTS^{\bullet+}$ radical scavenging activity of the EtOAc extract was observed to be 1.79-fold more potent ($p<0.05$) than that observed with the positive control Trolox (Figure 11E). The MeOH extract (Figure 11A) displayed comparable $ABTS^{\bullet+}$ radical scavenging ability to Trolox. The HW and HE extracts possessed lower free radical scavenging potential than Trolox (Figures 11B, D and E). Antioxidant activity has been reported in literature, with methanol and ethyl acetate root extracts of *T. sericea* displaying potent $ABTS^{\bullet+}$ radical scavenging activity with IC_{50} values of 75 µg/mL and 3 µg/mL, respectively.[326] Methanol and water extracts of *T. sericea* have been reported to be more potent than that of Trolox.[306] These findings support those of the current study, where IC_{50} values of 0.525 µg/mL and 0.387 µg/mL were obtained for the MeOH and EtOAc extracts, respectively (Table 10).

Similarly, in the DPPH assay, the MeOH extract (Figure 12A) was found to be significantly ($p<0.05$) more potent (1.5-fold) than Trolox (Figure 12E). No significant difference in the activity of the DPPH radical scavenging ability of the EtOAc extract (Figure 12C) as compared to Trolox was noted. The IC_{50} values obtained for the HW and HE extracts exceeded the highest concentration tested (Table 10; Figures 12B and D). In a previous study, hot water extracts of *T. sericea* leaves exhibited potent DPPH radical scavenging activity (IC_{50} = 4.25 µg/mL) whereas the methanol/acetone extract exhibited the highest free radical scavenging activity for the stem (IC_{50} = 4.51 µg/mL) and root (IC_{50} = 2.4 µg/mL) extracts.[327] The methanol root extract of *T. sericea* was found to posess potent radical scavenging activity in the DPPH assay with an IC_{50} of 14 µg/mL.[326] The findings of Masoko and Eloff[328] support the current results where the MeOH extracts were found to display strong antioxidant activity whereas the HE extract showed no antioxidant activity.

Table 10: $ABTS^{\bullet+}$ and DPPH radical scavenging activity of the crude extracts of *T. sericea* as compared to Trolox.

Free radical	IC_{50} (µg/mL)				
	MeOH	HW	EtOAc	HE	Trolox#
$ABTS^{\bullet+}$	0.525	2.990	0.387*	9.751	0.694
DPPH	9.080*	>100	12.660	>100	13.800

* $p < 0.05$ # positive control

MeOH = methanol; HW = hot water; EtOAc = ethyl acetate and HE = hexane

The crude extracts displayed protective effects against AAPH-induced oxidative damage in the cellular antioxidant assay (Figure 13). All the extracts displayed a dose dependent decrease in ROS and a protective profile against AAPH-induced damage. The extracts significantly ($p < 0.05$) reduced intracellular ROS as compared to the negative control, with the exception of the MeOH and HE extracts at concentrations > 3.2 µg/mL and the EtOAc extract at 0.1 µg/mL. None of the extracts were found to be more potent than the positive control, Trolox. The protective effects observed with the MeOH and EtOAc extracts in the cellular antioxidant assay is supported by the radical scavenging activity observed in the $ABTS^{\bullet+}$ and DPPH assays.

The HW and HE extracts which exhibited limited or no radical scavenging ability in the cell free assays displayed significant ($p < 0.05$) activity in the CAA assay. This may be due to the antioxidant activity of these extracts occurring via a different mechanism or a combination of different mechanisms which is not possible to evaluate in the cell free test models. Exposure of cells to AAPH generates a flux of peroxyl radicals, antioxidant compounds can protect H_2-DCF from oxidation (thus diminishing fluorescence) via different mechanisms, such as: (1) scavenging peroxyl radicals in the membrane diminishing lipoperoxidation, (2) reacting with AAPH avoiding intracellular peroxyl radical formation, (3) competing with H_2-DCF for oxidants such as ROS and reactive nitrogen species (RNS), (4) reacting with peroxyl radicals preventing the formation of other radicals, (5) inhibiting a redox pathway toward formation of ROS/RNS which oxidises H_2-DCF.[329]

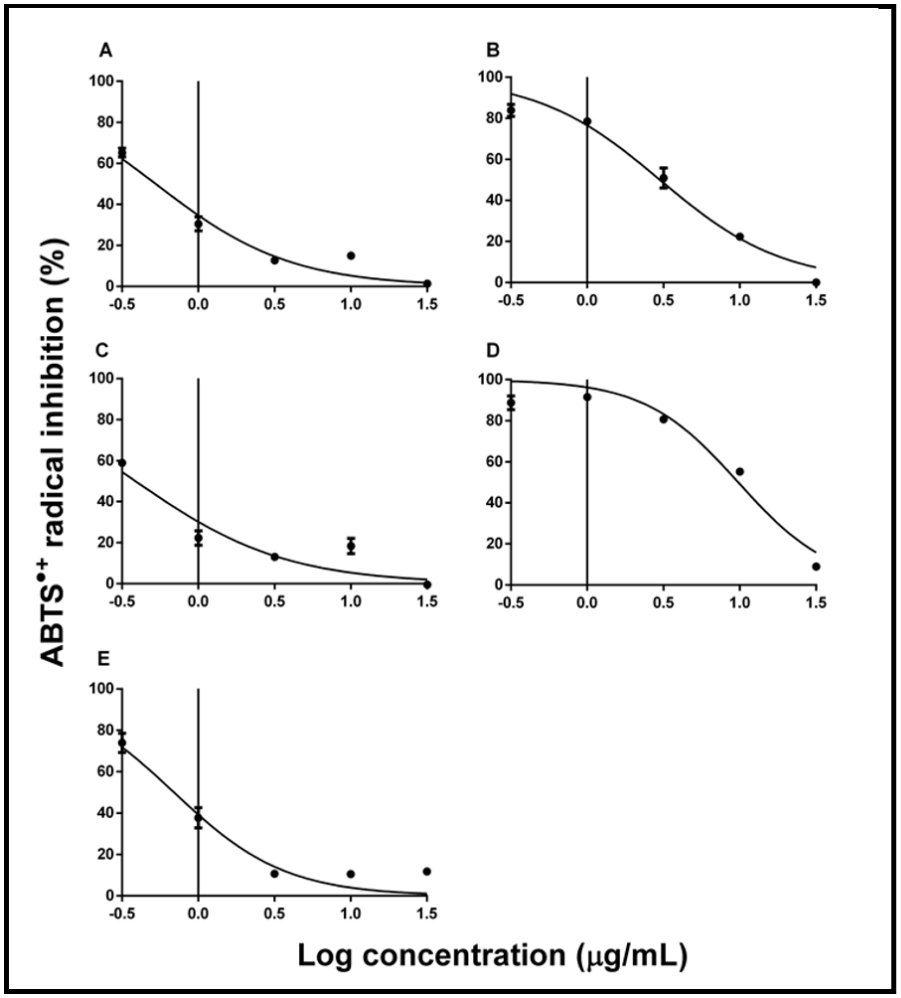


Figure 11: ABTS$^{\bullet+}$ radical scavenging ability of *T. sericea* extracts. A) methanol, B) hot water, C) ethyl acetate, D) hexane and E) Trolox (positive control).

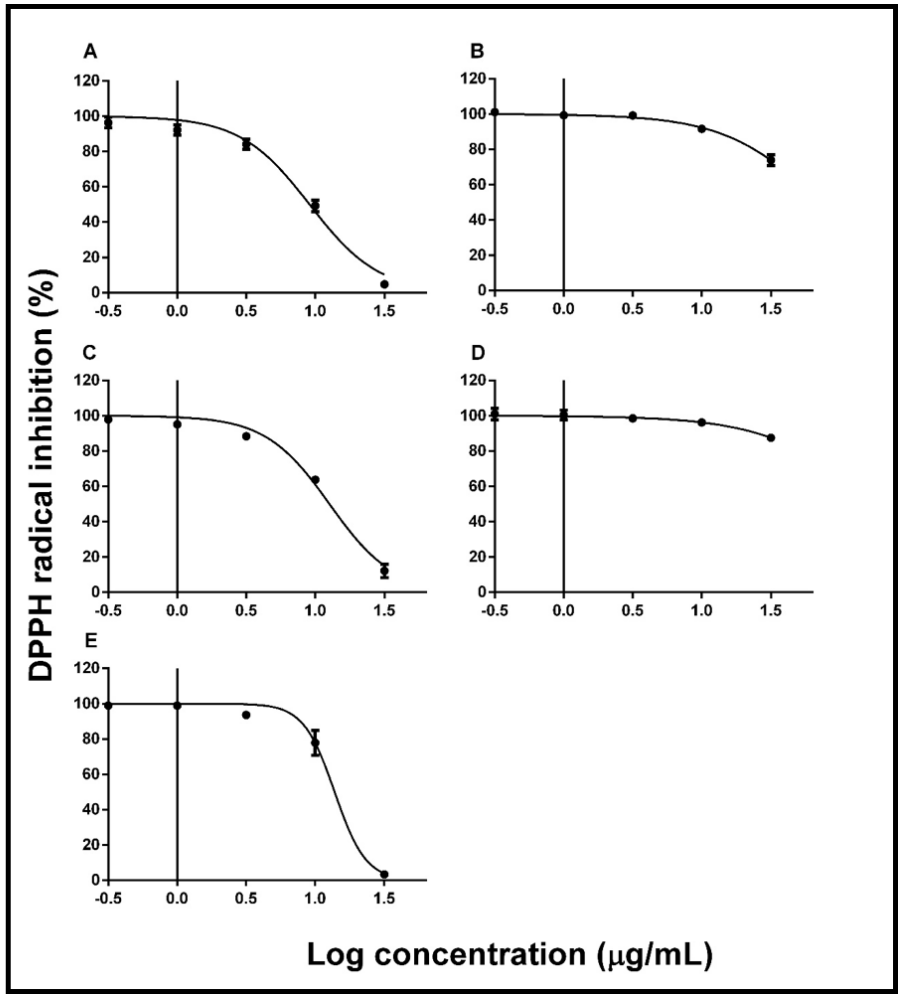


Figure 12: DPPH radical scavenging ability of *T. sericea* extracts. A) methanol, B) hot water, C) ethyl acetate, D) hexane and E) Trolox (positive control).

The MeOH, HW and EtOAc extracts at concentrations > 3.2 µg/mL resulted in a decrease in ROS (below the baseline levels) which could result in detrimental effects on the cells. The redox state of the cell is an intricate balance between the intrinsic antioxidants and ROS. If the system was not challenged with excess ROS, the excess antioxidants could push the cell into an oxidative state where cellular death could occur.[330] Since AAPH only induced a 13% increase in ROS as compared to the negative control, it could explain the reduction of ROS below baseline, as there would not have been excessive amounts of ROS to challenge the antioxidants introduced into the system via the plant extracts and Trolox.

To the best of the authors' knowledge, this is the first study to report on the protective effects of *T. sericea* extracts, against AAPH induced damage in a cellular antioxidant model

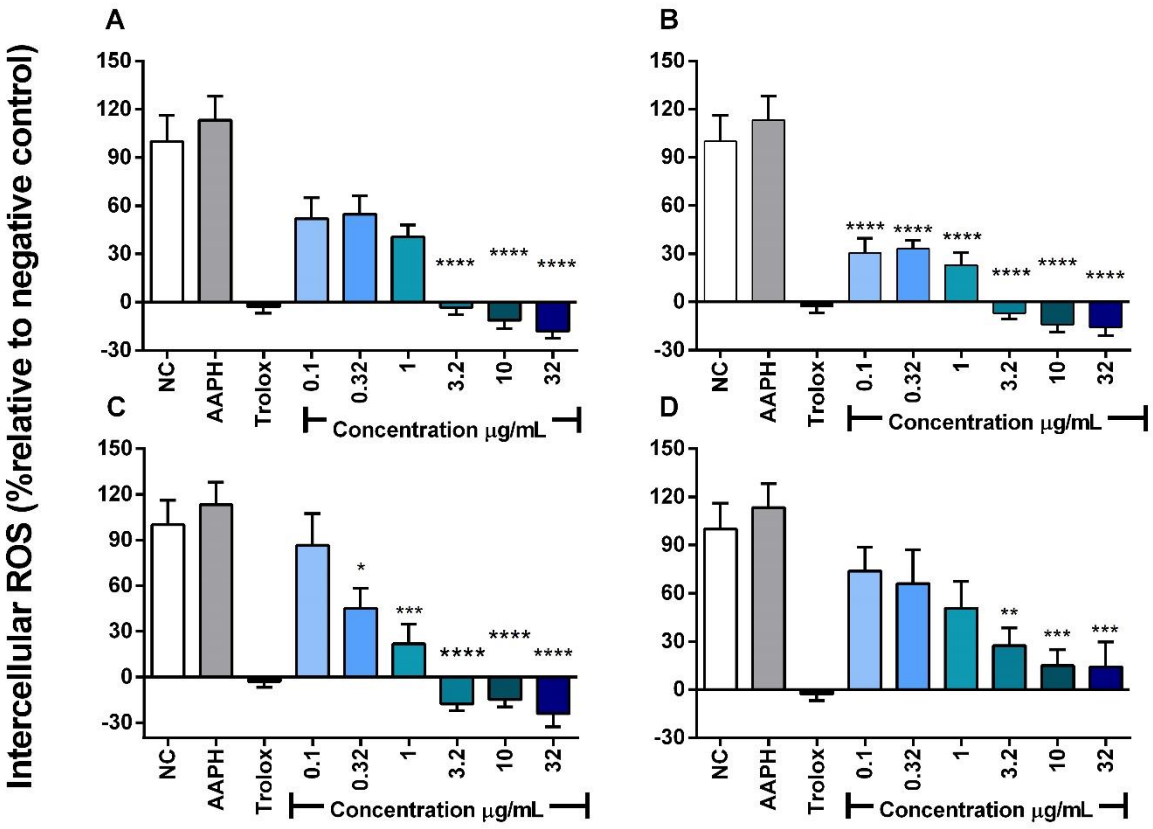


Figure 13: Protective effects of *T. sericea* crude extracts against oxidative damage in the SC-1 cell line after exposure to AAPH; A) methanol, B) hot water, C) ethyl acetate and D) hexane. NC: untreated * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$

The antioxidant activity of *T. sericea* has been attributed to flavonoids, phenolic acids, lignans (anolignan B) and saponins (sericoside). [297,306,331-334] These phytochemicals

were identified in the crude extracts using either TLC or UPLC-TOF-MS. The antioxidant activity attributed to phenolic acids is due to their stable radical intermediates, as well as their ability to donate hydrogen ions and electrons which stabilise other free radicals.[332,333] This is substantiated by the fact that an increased number of hydroxylated positions on the core structure usually corresponds with a more potent antioxidant activity.[332,333]

Phenolic acids previously identified in *T. sericea* include gallic acid, vanillic acid, 3,4-dihydroxybenzoic acid, caffeic acid, ferulic acid, syringic acid, 4-hydroxycinnamic acid, hydrocinnamic acid, benzoic acid and 4-hydrophenylacetic acid.[306] Hydroxycinnamic acid derivatives present in *T. sericea* are known to be potent antioxidants.[306] Caffeic acid, chlorogenic acid and ferulic acid have been reported to display wound healing activity by acting as free radical scavengers.[334-336]

Flavonoids have been reported to be scavengers of all known ROS.[333] Flavonoids express their antioxidant activity through mechanisms such as the scavenging of free radicals, chelation of metal (iron and copper) ions, and by inhibiting enzymes involved in free radical generation.[337] The wound healing ability of flavonoids have been attributed to their antioxidant properties.[331]

Anolignan B and sericoside[296] have been shown to possess antioxidant properties. Lupeol and mixtures of epicatechin-catechin and gallocatechin-epigallocatechin contribute to the antioxidant activity of the stem-bark of *T. sericea.*[338] Arjunic acid possesses strong antioxidant and free radical scavenging activity[339], and was detected in the MeOH and EtOAc extracts. These compounds individually or in combination may contribute to the antioxidant activity of *T. sericea*, which could be a result of the direct interaction between the antioxidant compounds present in the plant and ROS. The latter is reported to limit damage to cell membranes resulting in a protective effect against oxidative damage from free radicals.[340] Furthermore, the high number of hydroxylated positions on the core structures of these phytochemicals (Figure 10C and D) could be contributing factors to the antioxidant activity observed as an increase in these positions are usually correlated to more potent antioxidant activity as previously mentioned.[332,333]

Other species of *Terminalia* reported to possess antioxidant activity include: the bark of *T. arjuna*[341], *T. chebula*[342], *T. bellerica*[343] and *T. muelleri.*[344] High levels of glycosides may account for the antioxidant activity of the stem-bark of *T. arjuna*.[296] Bioactivity of the root extracts of *T. superba* has been attributed to the antioxidant ability of the phenolic acids in combination with other compounds.[345,346] The phenolic acids and glycosides detected in the extracts of *T. sericea* may be contributing factors to the antioxidant activity noted in this study (Table 8).

3.3. Antibacterial activity

3.3.1. Planktonic microorganisms

The disk diffusion assay was used as a screening method to determine whether or not extracts displayed antimicrobial activity. The MeOH, HW and EtOAc extracts displayed activity against all tested microbial strains, whereas the HE extract displayed activity only against *E. faecalis*, *P. aerginosa* and *V. parahaemolyticus* (Table 11). The MeOH, HW and EtOAc extracts displayed greater zones of inhibition as compared to their respective positive antibiotic controls in the case of *E. faecalis, E. coli* and *S. flexineri* (Table 11). The MeOH and EtOAc extracts displayed a larger zone of inhibition than the control ampicillin in the case of *B. cereus* and the EtOAc extract was found to be more possess a zone of inhibition greater than that of ciprofloxacin against *S. epidermidis* (Table 11). Even though the tested concentrations where higher than the antibiotic controls, it is important to note that crude extracts are complex mixtures of many phytochemical compounds, while the antibiotic controls are pure compounds. According to Rios and Recio[347], an MIC ≤ 1 mg/mL is considered as a good indication of microbial activity for a crude extract.[347]

The HE extract displayed antimicrobial activity against *E. faecalis*, *P. aerginosa* and *V. parahaemolyticus,* however these zones were smaller than those observed with the positive controls. The limited (small zones) or lack of inhibition observed with the HE extract could be due to the non-polar nature of the solvent or the lower concentration of the extract. It has been reported that the disk diffusion assay is not appropriate for the screening of non-polar substances due to differences in physical properties such as solubility, volatility and diffusion characteristics in agar.[27]

Table 11: Zones of inhibition of *T. sericea* crude extracts as determined by the disk diffusion assay.

Microorganism	Zone of inhibition (Mean (mm) ± SD)				
	MeOH (300 µg)	**HW (300 µg)**	**EtOAc (300 µg)**	**HE (1 µg)**	**Control (10 µg)**
Gram-positive					
Bacillus cereus	24.65 ± 1.55	18.01 ± 1.99	27.51 ± 1.52	NA	19.00 ± 1.35[a]
Enterococcus faecalis	24.08 ± 2.56	24.28 ± 4.19	24.77 ± 3.03	13.08 ± 2.24	21.56 ± 1.27[b]
Staphylococcus aureus	22.56 ± 1.41	20.26 ± 2.53	24.79 ± 1.85	NA	34.95 ± 2.59[c]
Staphylococcus epidermidis	14.52 ± 1.42	11.87 ± 1.67	19.64 ± 1.82	NA	16.33 ± 1.10[c]
Gram-negative					
Escherichia coli	22.22 ± 2.55	20.22 ± 1.78	23.75 ± 1.29	NA	15.59 ± 1.27[c]
Proteus vulgaris	19.48 ± 1.39	16.61 ± 1.47	18.80 ± 2.33	NA	24.01 ± 3.83[c]
Psuedomonas aeruginosa	23.40 ± 1.89	16.89 ± 1.37	23.66 ± 1.73	12.19 ± 1.41	34.89 ± 3.00[c]
Vibrio parahaemolyticus	24.82 ± 1.46	18.85 ± 1.16	24.73 ± 2.10	13.64 ± 1.34	29.59 ± 1.09[c]
Shigella flexineri	23.37 ± 1.71	16.09 ± 1.46	25.14 ± 1.01	NA	9.99 ± 4.35[b]

NA= no activity

Controls: a = Gentamycin; b = Ampicillin; c = Ciprofloxacin

MeOH = methanol; HW = hot water; EtOAc = ethyl acetate; HE = hexane

The current results are supported by findings where intermediate polarity extracts (MeOH and EtOAc) were found to exhibit greater zones of inhibition as compared to the non-polar extracts (petroleum ether) which displayed no activity.[348]

Ethyl acetate, methanol and aqueous root extracts of *T. sericea* have previously been found to inhibit the growth of *S. aureus*, *P. aeruginosa* and *E. coli* in the disk diffusion assay.[152] Similarly, inhibition of bacterial growth of *B. cereus*, *E. coli*, *P. vulgaris*, *P. aeruginosa*, *S. aureus* and *S. epidermidis* was reported for the methanol and water extracts of *T. sericea* leaves.[349]

In the broth microdilution assay the non-polar nature of the hexane did not pose a problem with diffusion (as compared to the disk diffusion assay) due to complete freedom in broth. The HE extracts displayed the lowest MIC values, showing inhibition of three microorganisms, *P. aeruginosa*, *S. epidermidis* and *V. parahaemolyticus* (Table 12). Although other extracts also inhibited bacterial growth, these were at concentrations >3 mg/ml (Table 12). The MBC values were found to be similar to or greater than the MIC values (Table 12).

Steenkamp *et al.*[291] reported MIC values of 1 mg/mL for the water and methanolic extracts of *T. sericea* bark against *S. aureus* and *Streptococcus pyogenes*, while no activity was noted against the Gram-negative bacteria up to concentrations of 4 mg/mL. These concentrations are lower than those noted in the present study, and are contradictory for Gram-negative bacteria, as the MeOH and HW extracts showed activity against *E. faecalis.* Similar to the current findings, methanolic and water extracts of the leaves were reported to have MIC values >1 mg/mL against *B. cereus, E. coli, P. vulgaris, S. aureus* and *S. epidermidis*, with no activity against *P. aeruginosa.*[349] However, in a later study, the authors from the same group reported MIC values <1 mg/ml against *B. cereus, E. faecalis, P. vulgaris, S. flexneri and S. aureus* for the dichloromethane-methanol and aqueous crude extracts of *T. sericea.*[350]

The antimicrobial activity of *T. sericea* extracts has been attributed to triterpene saponins, tannins, flavonoids and anolignan B.[271] The marker compound, anolignan B, isolated from the ethyl acetate root extract of *T. sericea*, has been reported to display good antimicrobial activity against *B. subtilis* (MIC: 3.8 µg/mL) and *E. coli* (MIC: 31 µg/mL).[271]

Table 12: Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values obtained for extracts using the broth micro-dilution assay.

Bacterial Strain	Crude extracts (mg/mL)								Control (µg/mL)	
	MeOH		HW		EtOAc		HE			
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-positive										
Bacillus cereus	6.25	6.25	12.5	25	3.13	3.13	-	-	1.5 [a]	1.5 [a]
Enterococcus faecalis	6.25	6.25	6.25	6.25	6.25	6.25	*	-	* [b]	* [b]
Staphylococcus epidermidis	12.5	12.5	6.25	6.25	3.13	3.13	1	1	1.5 [c]	1.5 [c]
Staphylococcus aureus	12.5	25	3.13	6.25	12.5	12.5	-	-	* [c]	* [c]
Gram-negative										
Escherichia coli	12.5	12.5	3.13	6.25	12.5	12.5	-	-	* [c]	* [c]
Proteus vulgaris	12.5	12.5	6.25	6.25	3.13	3.13	-	-	1.5 [c]	* [c]
Pseudomonas aeruginosa	12.5	12.5	6.25	6.25	12.5	12.5	1	1	1.5 [c]	3.13 [c]
Shigella flexineri	12.5	12.5	6.25	6.25	6.25	6.25	-	-	* [c]	* [c]
Vibrio parahaemolyticus	12.5	25	6.25	6.25	6.25	6.25	1	1	* [b]	1.5 [b]

*exceeds the highest concentration tested

- Not tested

Controls: a = Gentamycin; b = Ampicillin; c = Ciprofloxacin

MeOH = methanol; HW = hot water; EtOAc = ethyl acetate; HE = hexane

Arjunic acid, isolated from the ethyl acetate root extracts of *T. sericea*, has been reported to display inhibitory activity against Gram-positive and Gram-negative bacteria.[271] Arjunic acid, isolated from the bark of *T. arjuna*, has shown activity against *S. epidermidis* with a MIC of 125 µg/mL.[351] Anolignan b and arjunic acid were found to be present in both the EtOAc and MeOH extracts in this study, with the latter being highly abundant in the EtOAc extract. The lower MIC and MBC values obtained for the EtOAc extract could thus directly correlate to the abundance of arjunic acid. These phytochemicals may contribute to the activity of the MeOH and EtOAc crude extracts in the present study.

Alkaloids[352], phenolic acids (caffeic acid, chlorogenic acid and ferulic acid)[334-336] and flavonoids[331] are also reported to exhibit antimicrobial activity and may thus also contribute to the activity observed.

The differences observed in the antibacterial activity of the extracts of *T. sericea* in this study as compared to other studies, could be linked to a variety of factors which affect the concentration of phytochemicals found in plants. One such factor is annual rainfall which has been found to influence the antibacterial activity of *T. sericea.*[353] It was determined that plants collected during low annual rainfall seasons display more potent MIC values than those collected during high annual rainfall seasons.[353] The authors attributed this to the fact that water stress induces the production of secondary metabolites, such as phytoalexins and terpenes. Water stress can also reduce the activity of phytochemicals by the reduction of enzymatic activity or by inducing chemical conversion.[353] Another possible reason for the variation observed with the extracts could be due to the pH of ionisable phenolic or carboxylic compounds in dilutions, which also have the ability to change the activity noted.[347]

The concentrations of the extracts tested in this study are considerably higher than those used for conventional antibiotics, however it should be noted that crude extracts are a concentrated mixture of active compounds. Based on the proposal of Rios and Recio[347], that an MIC ≤1 mg/mL is a good indication of microbial activity for a crude extract, the activity noted for the HE extract (1 mg/mL) is considered to be an indication of promising antimicrobial activity which should be subjected to the isolation of the active phytochemical compound.

3.3.2. Biofilms

Since biofilms are the predominant mode of growth for bacteria in the majority of settings, including wounds, it is imperative to determine the effect that substances (such as the crude extracts tested in this study) have on these matrices. The biofilm inhibition assay was conducted only on those extracts which displayed MIC values ≤ 6.25 mg/mL. A 5 mg/mL cut off was selected for the purposes of this assay and results only reported as noteworthy if found to be below 1 mg/mL.

None of the extracts tested in the biofilm inhibition assay significantly reduced the growth of biofilms when compared to the negative control, but instead were found to enhance the growth of the biofilms (Figures 14 and 15). The HW and EtOAc extracts, although non-significantly, stimulated the growth of *E. coli* (Figure 14B), *P. vulgaris* (Figure 14C) and *S. flexineri* (Figure 15B). The EtOAc extract significantly ($p < 0.05$) enhanced the growth of *B. cereus* (Figure 14A), *P. aeruginosa* (Figure 14D) and *V. parahaemolyticus* (Figure 15C). The HW extract was found to stimulate the growth of *P. aeruginosa* (Figure 14D), *S. aureus* (Figure 15A) and *V. parahaemolyticus* (Figure 15C) biofilms, significantly ($p < 0.05$). No dose-dependent trends were observed with any of the extracts.

The HE (0.16 mg/mL) and MeOH (0.08 mg/mL) extracts reduced the growth of the *P. aeruginosa* and *P. vulgaris* biofilm growth by 33.2% (Figure 14D) and 54.6%, respectively (Figure 14C). The positive controls were not found to be significantly different to the negative control, even though in some instances a marked reduction in bacterial growth was observed. To the best of the author's knowledge this is the first study to report the effects of *T. sericea* on the growth of biofilms.

The slight inhibition observed with the HE and MeOH extracts could be attributed to ellagic acid and tannic acids or derivatives thereof that have been previously detected within the plant.[354] Ellagic acid and tannic acid have been found to reduce biofilm formation of clinical strains of *E. coli* by 44–80% and 22–26%, respectively.[355] Also, tannin-rich fractions of *T. catappa* leaves were found to decrease biofilm growth of *Chromobacterium violaceum* and *E. coli* through the inhibition of phenotypic expression of quorum sensing molecules.[354]

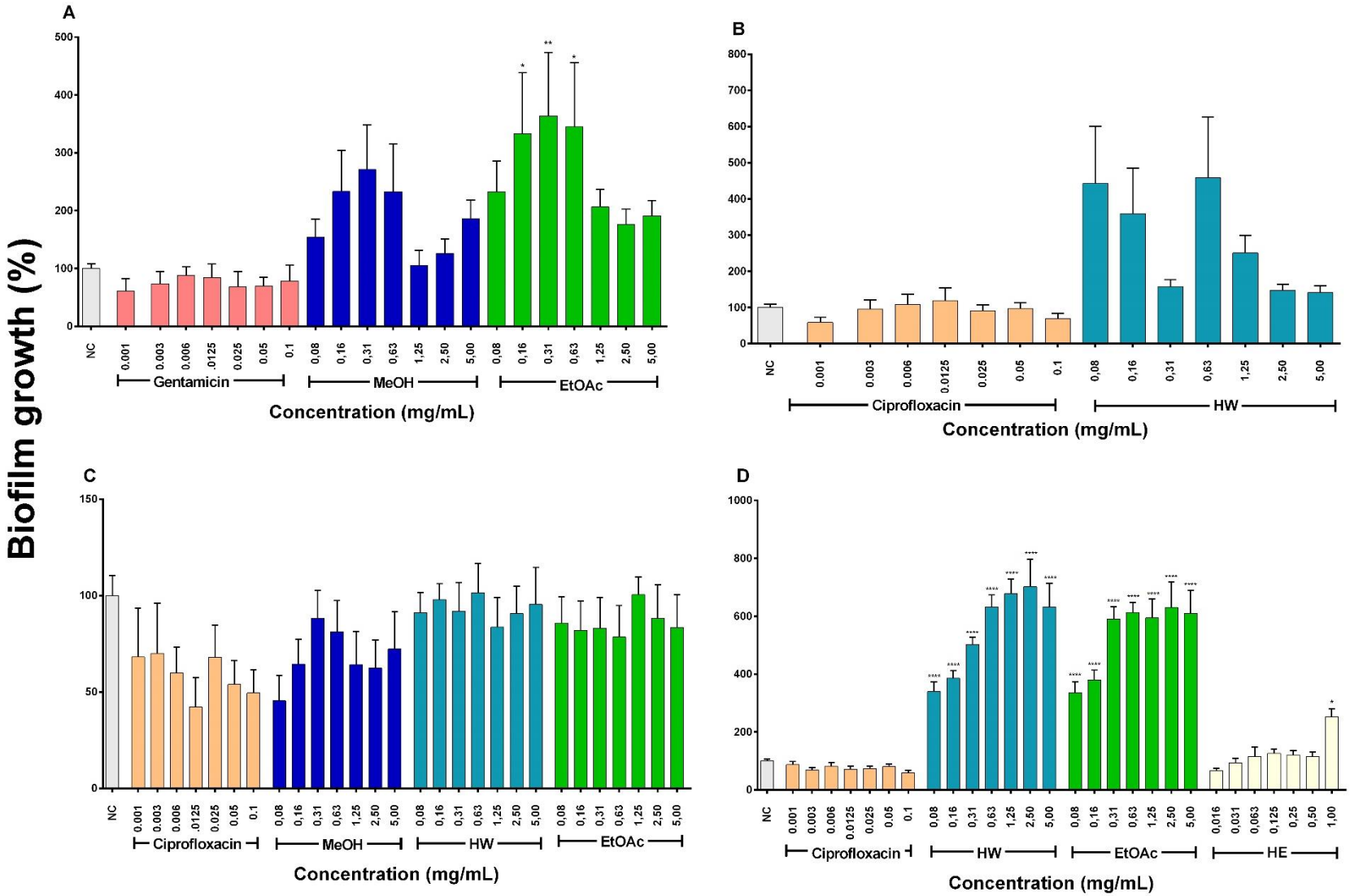


Figure 14: Effect of *T. sericea* extracts on biofilm growth. A) *B. cereus*; B) *E. coli*; C) *P. vulgaris* and D) *P. aeruginosa*. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.00$; **** $p \leq 0.0001$. MeOH = methanol; HW = hot water; EtOAc = ethyl acetate; HE = hexane

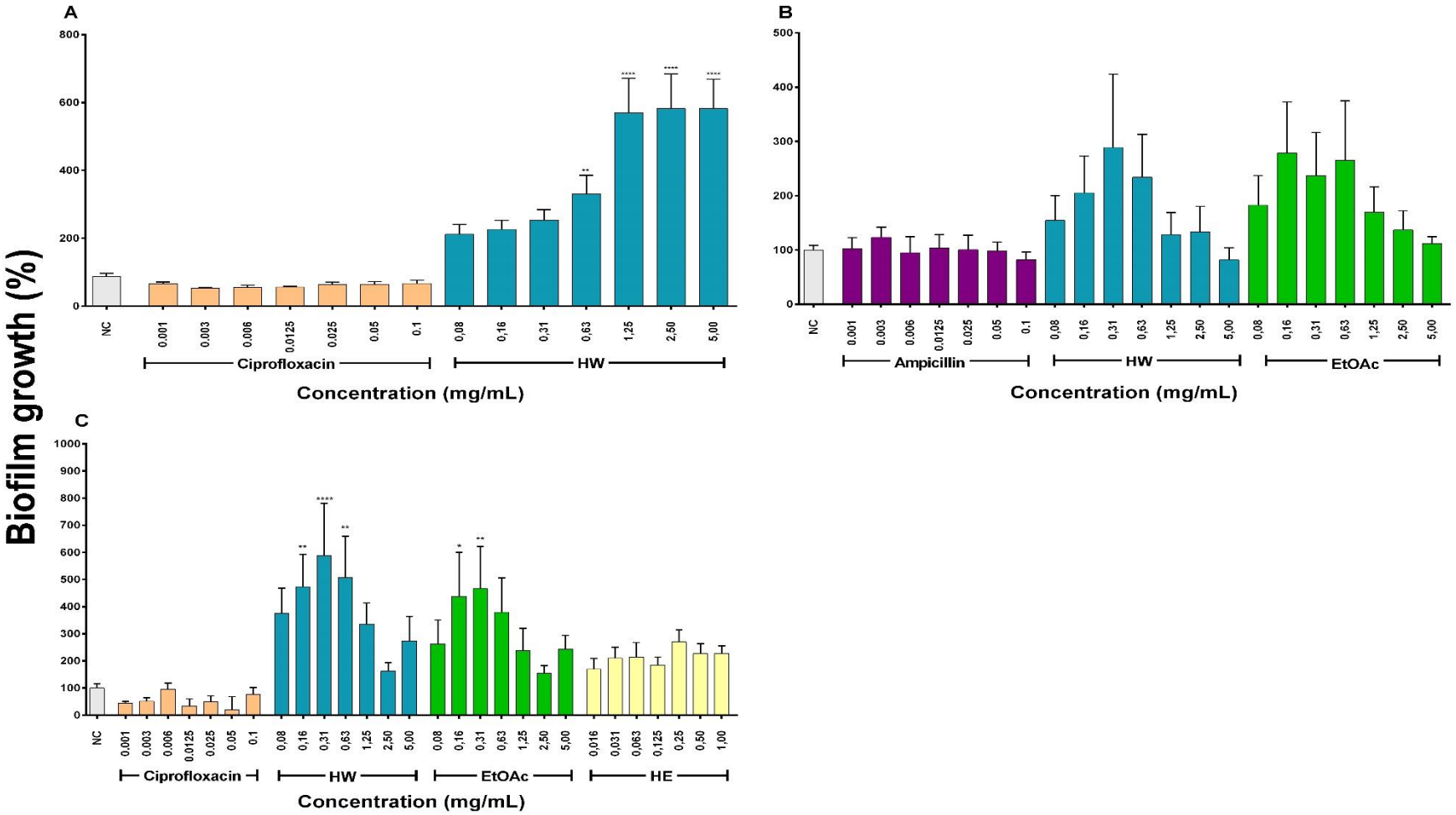


Figure 15: Effect of *T. sericea* extracts on biofilm growth. A) *S. aureus;* B) *V. parahaemolyticus* and C) *S. flexineri. * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001*. MeOH = methanol; HW = hot water; EtOAc = ethyl acetate; HE = hexane

The stimulation of biofilm growth by plant extracts has also been reported in literature. Root extracts of *Arabidopsis thaliana* have been found to stimulate *B. subtilis* biofilm formation. This was ascribed to the polysaccharides; arabinogalactan, pectin, and xylan which served as growth signals.[356] These authors found that the polysaccharides were used by the bacteria as a source of sugars for the synthesis of the matrix exopolysaccharide.[356] The latter may explain the stimulation noted in the biofilm formation assay in the present study.

The biofilm stimulatory effects of the extracts at the tested concentrations suggest that the extracts are not strong anti-biofilm substances and could deter treatment if a mature biofilm has already formed. However, as selected extracts displayed activity against planktonic bacteria, if treatment were to occur before bacterial infection were to set it in, it could assist in killing planktonic bacteria before it progresses to form a biofilm.

3.4. *In vitro* cytotoxicity

The effects of the crude extracts on the density of fibroblast (SC-1) cells and endothelial (EA.hy926) cells after 24 and 72 h are depicted in Figures 16 and 17, respectively. The crude extracts did not reduce cell density by more than 50% in any of the cell lines after 24 h of exposure (Figures 16 and 17). After a 72 h exposure period, the HW and HE extracts still did not induce cytotoxicity in either of the cell lines (Figures 16B, D and 17B, D).The MeOH and EtOAc extracts, however, resulted in a decrease in cell density after a 72 h exposure period. The IC_{50} values (Table 13) were low and a time dependent killing effect was noted (Figures 16A, C and 17A, C). Based on the results obtained, it appears that cytotoxicity is confined to the intermediate polarity solvents (MeOH and EtOAc). The cytotoxicity induced by the extracts were substantially lower than that of saponin, the positive control (Table 13).

At sub-toxic concentrations the HW, EtOAc and HE extracts appeared to induce cellular proliferation, which could be explained by a cell proliferative hormetic effect. Hormetic dose-response effects are an adaptive response exhibited by cells induced with cytotoxic agents, and are often characterized by stimulation at low doses and inhibition at high doses.[357] These effects occur through mechanisms such as the enhancement of DNA repair and antioxidant capacity, which could explain the current results observed.[358]

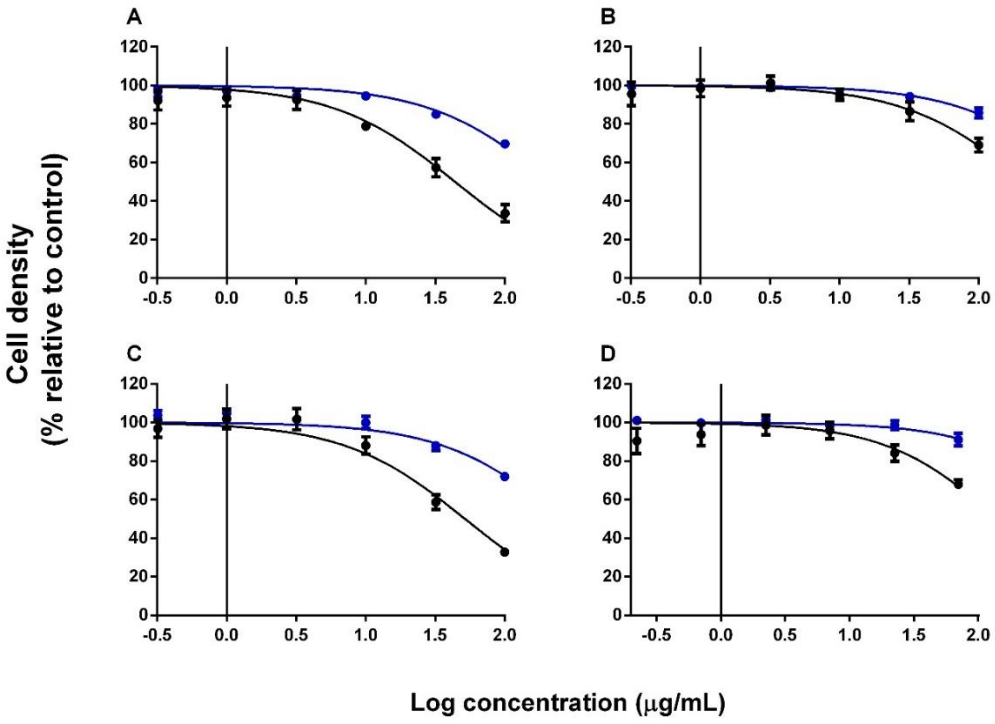


Figure 16: Effect of *T. sericea* crude extracts on the cell density of SC-1 cells after a 24 h ( ) and 72 h ( ) exposure period. A) methanol, B) hot water, C) ethyl acetate and D) hexane.

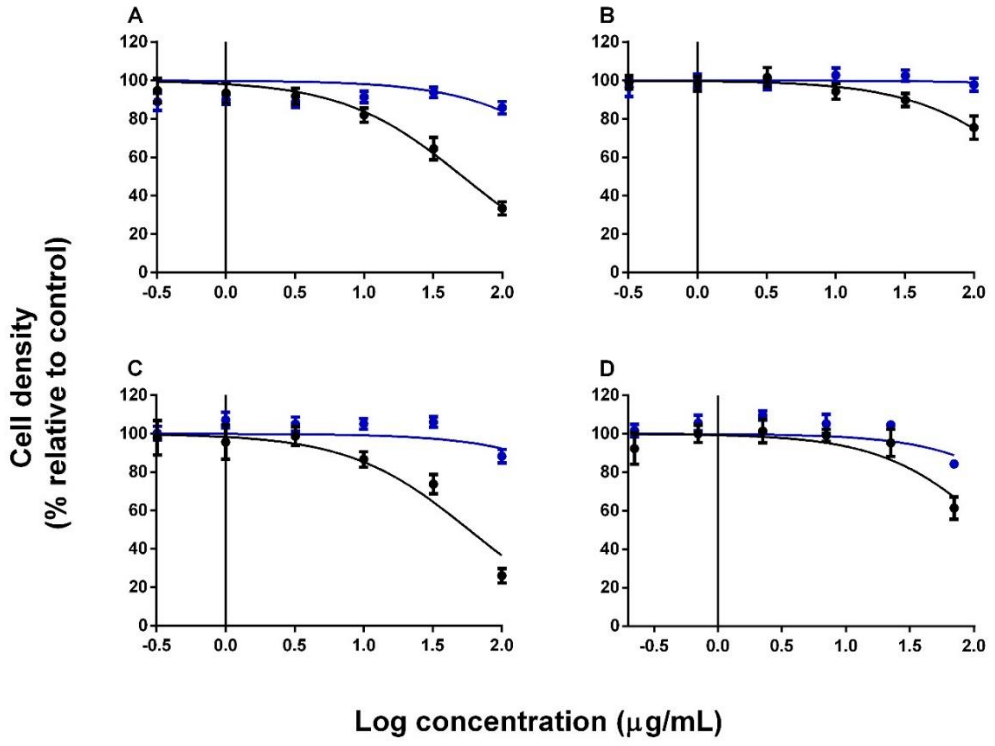


Figure 17: Effect of *T. sericea* crude extracts on the cell density of EA.hy926 cells after a 24 h ( ) and 72 h ( ) exposure period. A) methanol, B) hot water, C) ethyl acetate and D) hexane.

Table 13: Half-maximal inhibitory concentrations (IC_{50}) obtained for *T. sericea* crude extracts in the SC-1 and EA.hy926 cell lines after 24 h and 72 h exposure.

Cell line	IC_{50} ± SEM (µg/mL)				
	MeOH	HW	EtOAc	HE	Saponin
SC-1 (24 h)	>100	>100	>100	>100	0.048
SC-1 (72 h)	33.77 ± 1.127	>100	53.04 ± 1.132	>100	0.057
EA.hy926 (24 h)	>100	>100	>100	>100	0.111
EA.hy926 (72 h)	51.82 ± 1.054	>100	57.83 ± 1.085	>100	0.007

The roots of *T. sericea* are reputed to be poisonous.[100] It has been reported that the roots of *T. sericea* alone contain more cytotoxic agents than the other plant parts or when different parts are combined.[152,348,359] Since a combination of roots and leaves were used in this study, the possibility exists that the cytotoxicity of the roots were reduced by the less toxic nature of the leaves.

Previous studies conducted using various solvent extracts of *T. sericea* have reported cytotoxic activity for selected parts of the plant. In a study by Moshi and Mwambo[152], half maximal lethal concentrations (LC_{50}) of 16.4 µg/mL, 16.9 µg/mL, 5.4 µg/mL were obtained for the MeOH, EtOAc and aqueous root extracts of *T. sericea*, respectively, in the brine shrimp lethality test. The crude aqueous extract of *T. sericea* pods and roots had a half-maximal inhibitory dose (ID_{50}) of 24.0 µg/mL after a 7 day incubation period in monkey kidney cells.[359] Contradictory to the results found in the present study, a more pronounced cytotoxic effect was noted in these studies. The methanol leaf and root extracts (25 µg/mL) of *T. sericea* have been found to reduce T 24 (bladder cancer) cell growth to 61.0% and 29.3%, HeLa (cervical cancer) cell growth by 84.0% and 58.2% and MCF-7 (breast cancer) cell growth by 55.4% and 53.2%, respectively.[360] The potent cytotoxicity noted against cancerous cell lines but absence thereof noted in primary cell lines, could be indicative of an inherent cancer selectivity by *T. sericea*.

Cytotoxic activity observed with the higher concentrations of the MeOH and EtOAc extracts could be attributed to certain phytochemicals. Saponins and glycosides are known to have cytotoxic properties.[361-364] The cytotoxic effects observed with saponins appears to be due to their ability to induce apoptotic processes, cause cell cycle arrest,

stimulate autophagic cell death and disintegrate the cytoskeleton.[365] 3'5'-dihydroxy-4-(2-hydroxy-ethoxy) reservatrol-3-O-β-ruinoside is a hydroxystilbene glycoside present in the roots of *T. sericea.*[310] Stilbene glycosides have previously been shown to be cytotoxic.[362,363] Glycosides induce toxicity by the inhibiting oxidative phosphorylation in the outer membranes of the mitochondria.[366]

Caffeic acid, a phenolic acid previously identified in the roots of *T. sericea*[306], has been found to result in a proliferative effect in mouse fibroblasts after a 32 min pre-treatment.[367] However, in another study conducted on mouse lung fibroblasts, cytotoxicity (> 20% viability) was only observed after 72 h of exposure at concentrations > 50 µg/mL of caffeic acid.[368] This is similar to the trend noted with the MeOH and EtOAc extracts in this study where cytotoxicity was only apparent after 72 h exposure.

Punicalagin has been reported to display anti-proliferative activity against human oral (KB, CAL27), colon (HT-29, HCT116, SW480, SW620) and prostate (RWPE-1, 22Rv1) tumour cells.[369] Punicalagin was identified in the MeOH, HW and EtOAc extracts in the present study. Since the HW extracts did not display cytotoxicity, the cytotoxic activity noted is probably not entirely attributed to this phytochemical acting in its individual capacity but rather as a result of a synergistic effect with other compounds.

3.5. Cell proliferation and migration

In vitro wound healing models allow insight into the biochemical and physiological processes induced by test compounds/agents.[16] These models are relevant in the study of cell-cell and cell-matrix interaction to mimic cell migration during wound healing and can employ single cell systems, three dimensional systems, multicellular systems or organ cultures in assessing the wound healing properties of agents or compounds.[16] The fibroblast proliferation assay was employed to determine the effects of the crude extracts on *In vitro* cellular proliferation and the scratch assay utilised to evaluate effects on cellular migration. For this part of the study, a maximum concentration of 32 µg/mL was used in the fibroblast proliferation assay and 10 µg/mL for the scratch assay.

The positive control (15% FCS) enhanced proliferation, inducing a 2-fold increase in cell density as compared to the negative control (Figure 18). The EtOAc crude extract

at 1 µg/mL displayed a significant ($p \leq 0.05$) increase in SC-1 fibroblast cell density as compared to the negative control (Figure 18C). Whereas all other crude extracts in the concentration range 0.32-10 µg/mL exhibited non-significant increases in cell density compared to the negative control (Figure 18A-D). Of particular interest, the HW extract at 1 µg/mL increased cell density by 30.4% as compared to the negative control (Figure 18B). All the extracts at 32 µg/mL resulted in a reduction in cell density, which was more pronounced after treatment with the MeOH and EtOAc extracts (Figure 18). This finding correlates with what was found in the cytotoxicity assays.

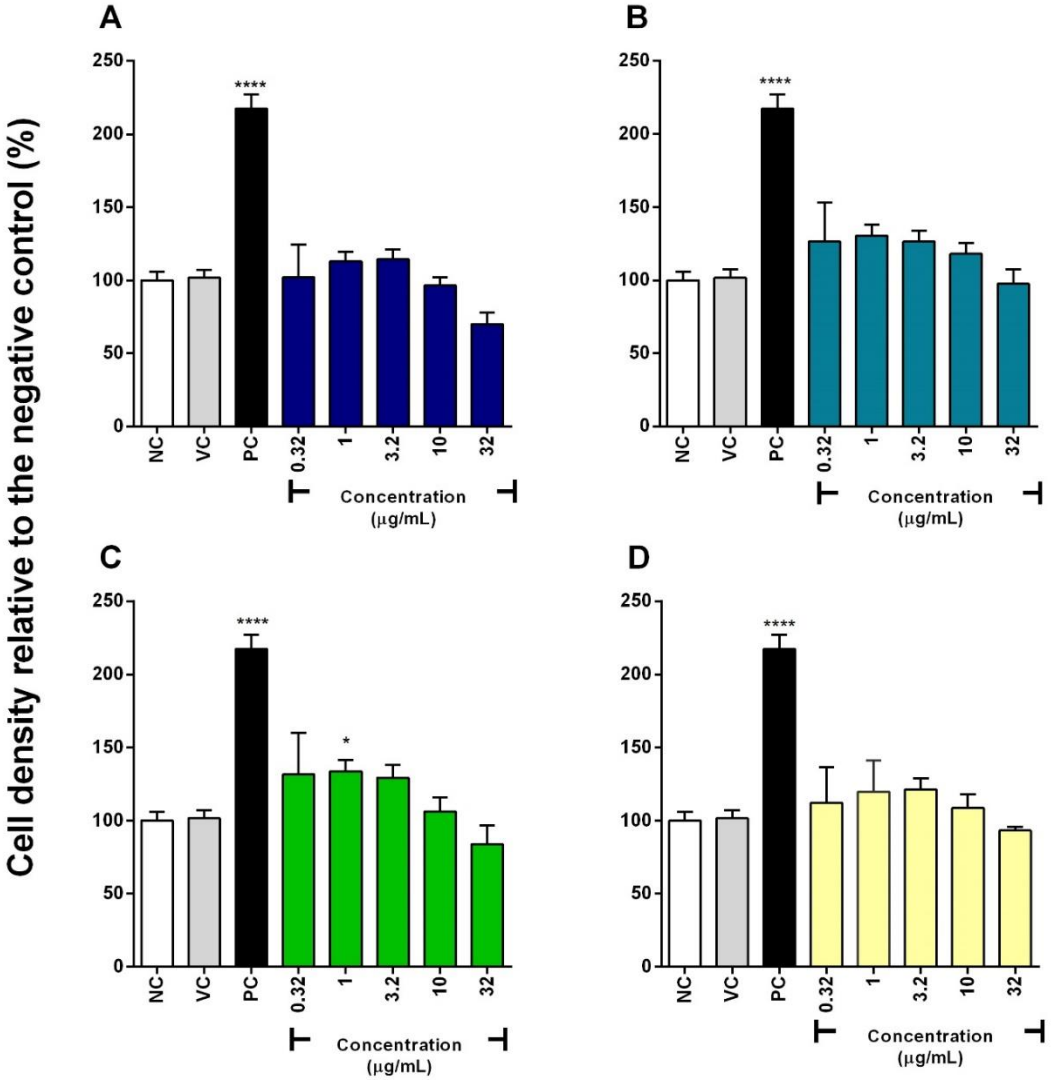


Figure 18: Effect of *T. sericea* extracts on SC-1 fibroblast proliferation; A) methanol; B) hot water; C) ethyl acetate and D) hexane; PC: 15% FCS *$p \leq 0.05$; ***$p \leq 0.001$

Water and MeOH extracts of *T. sericea* have been noted to have no effect on the growth of fibroblasts up to concentrations of 1 mg/mL.[291] The latter finding is contradictory to the results observed in the present study, as some increase in cell density was noted, albeit at much lower concentrations.

All crude extracts enhanced the migration of SC-1 fibroblasts when compared to the negative control (Figures 19 and 20A). Although none of the crude extracts exhibited a significantly greater induction of migration than the positive control (PDGF), the hexane extracts (Figures19D and 20F) at 3.2 and 10 µg/mL resulted in an 86.43% and 80.79% closure of the simulated wound, respectively, as compared to the 74.3% closure of PDGF (Figures 19 and 20B). The lowest concentration of all extracts tested (1 µg/mL) had the greatest effect on migration (Figures 19A-D and 20C-E). As the concentration of the extracts increased, a reduction in cell migration was observed i.e. dose dependent effect.

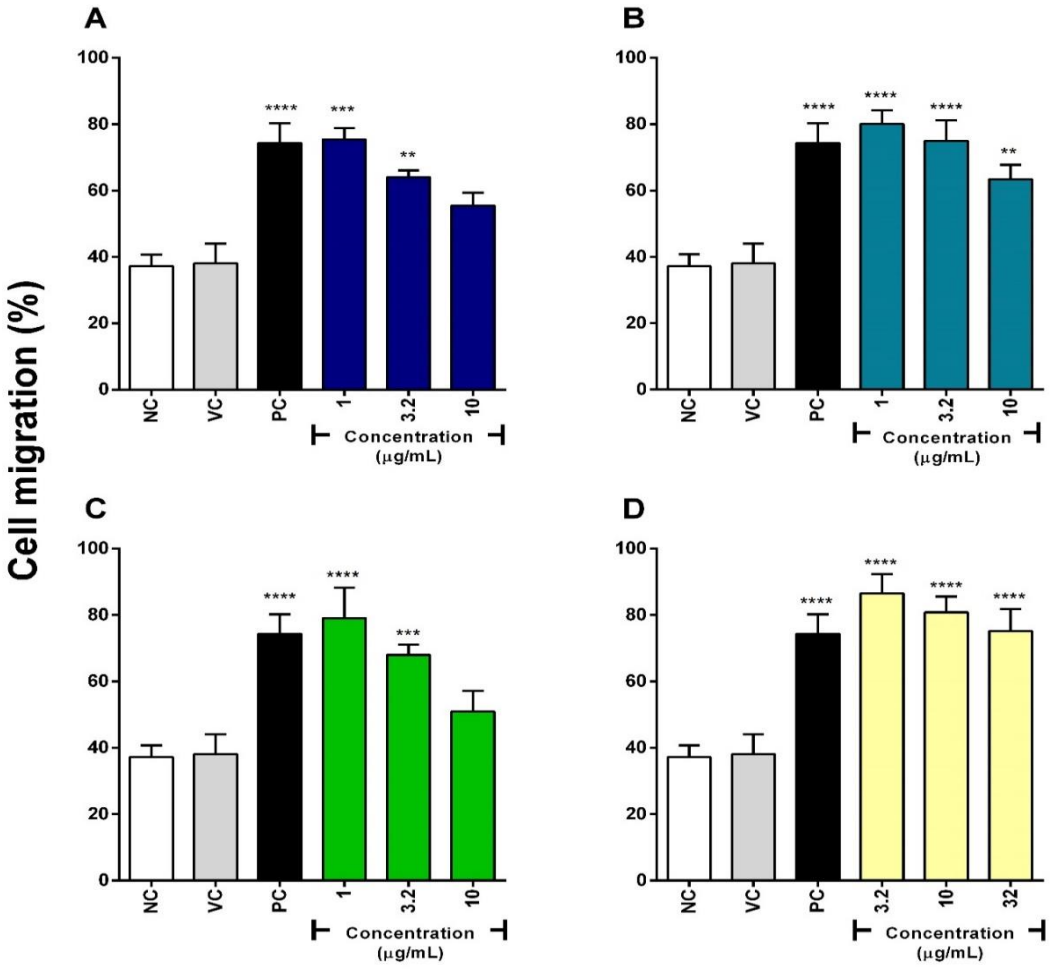


Figure 19: Effects of *T. sericea* crude extracts on cell migration in the SC-1 cell line: A) methanol; B) hot water; C) ethyl acetate and D) hexane extracts; PC: PDGF **p ≤ 0.01; *** p ≤ 0.001; ****p ≤ 0.0001

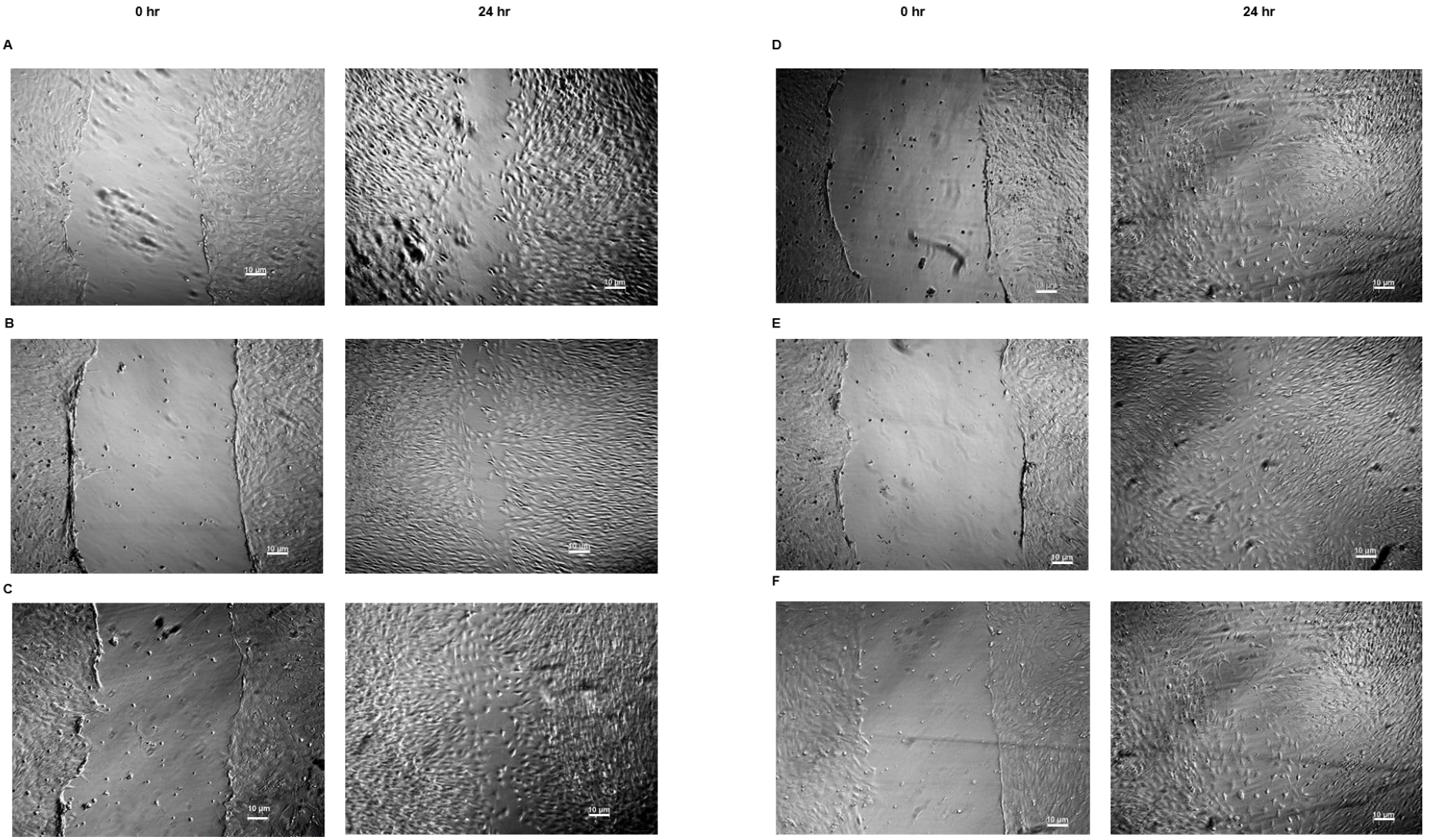


Figure 20: Representative phase contrast microscopy images displaying migration of cells in the SC-1 cell line after a 24 h exposure period. A) NC; B) PDGF; C) methanol (1 µg/mL); D) hot water (3.2 µg/mL); E) ethyl acetate (1 µg/mL) and F) hexane (3.2 µg/mL). White bar represents 10 µm.

Similar to the trend observed in the SC-1 cell line, most extracts significantly ($p < 0.05$) enhanced cell migration in the EA.hy926 cell line as compared to the negative control (Figures 21 and 22A-F). The HE and HW extracts, at all tested concentrations, enhanced cell migration to a greater extent than PDGF (Figures 21B,D and 22D,F).

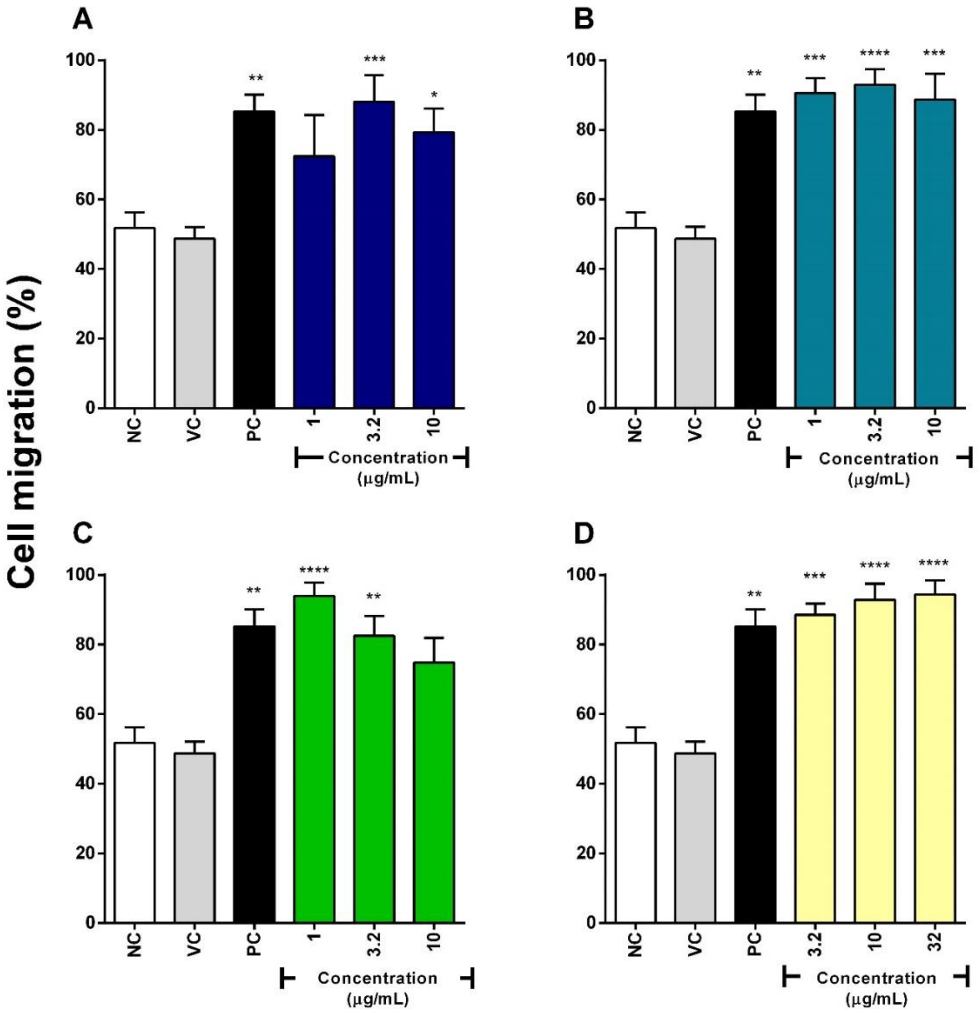


Figure 21: Effects of *T. sericea* crude extracts on cell migration in the EA.hy926 cell line: A) methanol; B) hot water; C) ethyl acetate and D) hexane extracts; PC: PDGF *$p \leq 0.05$; **$p \leq 0.01$;***$p \leq 0.001$;****$p \leq 0.0001$

The HE extract (32 µg/mL) had a prominent migratory effect, resulting in a 94.34% closure of the simulated wound, this was followed by a closure of 93.91% from the EtOAc at 1 µg/mL extract and a 93% closure by the HW extract at 3.2 µg/mL (Figures 21B-D and 22D-E). The positive control (PDGF) resulted in an 85.27% closure of the simulated wound (Figures 21 and 22B). A dose dependant increase and decrease in cell migration was observed for HE and EtOAc crude extracts, respectively.

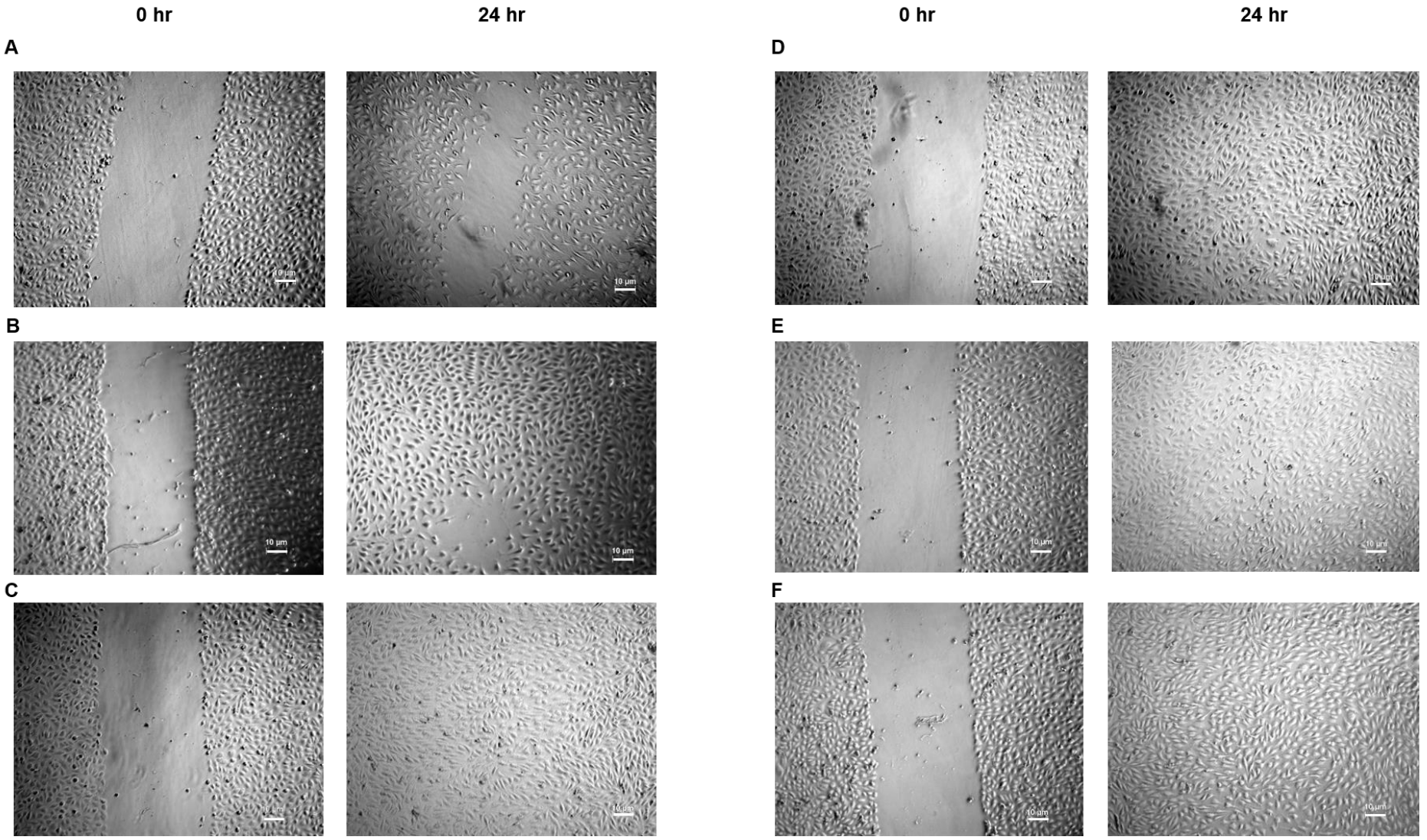


Figure 22: Representative phase contrast microscopy images displaying migration in the EA.hy926 cell line after a 24 h incubation period. A) NC; B) PDGF; C) methanol (3.2 µg/mL); D) hot water (3.2 µg/mL); E) ethyl acetate (1 µg/mL) and F) hexane (32 µg/mL). White bar represents 10 µm.

Certain phytochemicals have been shown to have growth factor-like activity. These compounds may induce similar proliferative pathways, such as the activation of the Na^+/H^+-exchanger (which controls the speed and directionality of migrating cells) and the activation of the Nck family of domain adaptors (which regulates cytoskeletal reorganization and chemotaxis stimulated by PDGF).[51] Flavonoids have been reported to display growth factor stimulatory activities.[199] Some saponin compounds have been found to exert wound healing effects through growth factor-like activity or stimulation of growth factor expression.[370] As observed in the present study, all extracts stimulated cellular migration and replication and this effect may be via the activation of the cellular migratory and replication pathways.

The migratory activity observed may also be linked to the antioxidant activity as it has been associated with cell migration through the Rho-associated guanosine triphosphate protein (RacGTP) signal-transduction pathway.[371] Rac is a small GTPase protein involved in cell signalling and motility of migrating cells.[372] Resveratrol, ferulic acid, phloretin and tetrahydro-curcuminoids, all of which possess antioxidant activity, have been found to enhance migration of oral fibroblasts in the scratch assay through the upregulation of RacGTP.[371] The compounds, ferulic acid and resveratrol-3-O-β-rutinoside (a resveratrol derivative) have been identified in *T. sericea*[306,310] and the migratory activity noted may therefore be due to these compounds. Antioxidants may further contribute to cell proliferation and migration through alterations to reduction/oxidation (redox) reactions in the cellular environment.

The cellular redox environment has been found to affect cellular signal transduction, DNA and RNA synthesis, protein synthesis, enzyme activation, regulation of the cell cycle, ligand binding, DNA binding and nuclear translocation which ultimately leads to cell proliferation or death.[330] The cellular redox environment is a balance between the production of ROS and its removal by antioxidant enzymes and small molecular weight antioxidants.[373] The increase in antioxidant activity by plant compounds has been found to shift the cellular redox environment to a reducing state, which favours cellular proliferation and migration.[374,375] This is supported by the results of the CAA assay (Figure 13). At the lower extract concentrations (1 and 3.2 µg/mL) where antioxidant activity was not as pronounced as with the higher concentrations, enhanced cell migratory (Figure 13) and proliferative (Figure 13) effects are observed.

β-sitosterol and β-sitosterol glucosides, which have been identified in the *Terminalia* genus, have been found to promote endothelial cell proliferation and angiogenesis in their reduced state, but have been found to induce cell death in their oxidised state.[376, 377] Phenolic compounds, including caffeic acid, have been reported to enhance proliferation of 3T3-L1 mouse fibroblasts. LC-MS/MS analysis identified the presence of catechin monomers and dimers in the intracellular compartment, indicating that phenolic compounds had incorporated themselves into the cells to exert proliferative and antioxidant effects.[367] Caffeic acid has previously been identified as one of the phenolic acids present in *T. sericea.*[306] Phytochemicals in *T. coriacea*, which are known to stimulate cellular proliferation, include; anthraquinone glycosides, flavonoids and tannins.[296,378] These phytochemicals are also present in *T. sericea*, and therefore could be responsible for the migration and increased cell density observed. Due to the high antioxidant activity of *T. sericea*, it is likely that they may have contributed to the increased cellular migration at lower concentrations.

The wound healing activity of plants belonging to the *Terminalia* genus has been extensively reported in literature. It has been postulated that wound healing effects of extracts from the *Terminalia* species may involve the synergistic action of multiple components via several pathways.[296] *T. arjuna*[126], *T. chebula*[379 380] and *T. coriacea* [296] have been found to accelerate the wound healing process. *T. arjuna* bark extracts have been reported to enhance the tensile strength and increase the rate of epithelialisation of incision wounds in a rat model.[381] The wound healing activity of this plant has been attributed to tannins, which were found to enhance cellular growth and proliferation, and assist in matrix formation.[126] Similarly, *T. chebula* enhances extracellular matrix formation through the enhancement of protein synthesis and deposition of matrix proteins in granulation tissues.[379] The tannin extracts of *T. chebula* have demonstrated angiogenic activity by increasing the number of new capillaries during the inflammatory phase of wound healing, as well as displaying an increased degree of wound contraction in granulation tissue.[380] The enhancement of *in vitro* wound healing observed with the extracts of *T. sericea* in this study can be attributed to the multiple actions of the various phytochemicals identified in the plant on the various stages of the wound healing process.

3.6. The effect of *T. sericea* on wound healing

The haemostatic phase of the wound healing process is defined by the constriction of blood vessels and the formation of a haemostatic plug.[18] Flavonoids and tannins have been reported to demonstrate haemostatic action by decreasing bleeding time, precipitating proteins to form vascular plugs and causing vasoconstriction.[382] Tannins present in *Terminalia* spp. have been implicated to be responsible for enhancing the wound healing effect[296], which could be attributed to its effects on the haemostatic phase of wound healing.[296] As these compounds were detected in the extracts, it is possible that they are responsible for enhancing wound healing by the proposed mechanism.

The inflammatory phase of wound healing is crucial, as a prolonged inflammatory phase can result in the progression to a chronic wound.[383-385] Inflammation is accompanied by high amounts of ROS at the wound site, which assists in wound debridement and antimicrobial defence.[3] Excessive amounts of ROS can result in oxidative damage to cells and tissues.[3] The ability of an extract to scavenge free radicals contributes to its role in wound healing.[22] Antioxidant compounds aid in the removal ROS and protection of cells via the removal of inflammatory products.[22]

Punicalgin has been identified for its role in wound healing via its anti-inflammatory action through the inhibition of inflammatory factors such as nitric oxide (NO), PGE2, IL-1β, IL-6, TNF-α and NF-κB activation in macraophages.[386] Sericoside, the tritepeniodal saponin present in *T. sericea* has been reported to possess anti-inflammatory and antioxidant activity.[291,387] Saponins have been found to inhibit inflammatory mechanisms in the early phases of wound healing.[388] Coumarins and alkaloids have been implicated in wound healing through antioxidant effects.[173,296]

Another factor that can prolong the inflammatory phase is the infection of the wound by microorganisms.[3] Saponins, flavonoids and other phenolics have been speculated to contribute to wound healing by acting as antimicrobial substances through their detergent ability to remove grease, dirt and bacteria from tissue.[3] Antioxidant and antibacterial activity observed with the extracts could explain their role in this phase of wound healing.

The proliferative and remodelling phases are characterised by the migration of fibroblasts to the wound site where they initiate remodelling through collagen production.[8] Saponins have been reported to promote re-epithelisation of skin wounds in a mouse model, by enhancing collagen synthesis in skin fibroblasts through the phosphorylation of the Smad-2 protein.[388] Saponins enhance wound healing by stimulating of growth factor release.[370] Punicalagin inhibits mitogen activated protein kinases (MAPKs), which have been found to play a crucial role in the progression of cell migration.[389] Alkaloids have been found to accelerate the wound healing process by stimulation of chemotaxis in fibroblasts.[303,390] As these compounds were detected in the extracts, it is possible that they are responsible for the migratory activity noted and may contribute to proliferation and migration.

The phytochemical compounds in each extract appear to have acted synergistically or additively to achieve enhanced effects in one aspect, but also to have acted antagonistically to reduce effects in others. Such as the reduced cytotoxic effects noted with the HW and HE extracts and the enhanced antioxidant effects noted with the MeOH and EtOAc extracts. The effects observed in the current study is most likely due to combinational effect of the phytochemical matrix.

An ideal wound healing agent would maintain a wound environment free of bacteria that promotes wound healing, i.e. not cytotoxic, improves oxidative status and promotes cellular proliferation and migration. The extracts the showed the most promise were the EtOAc and the HE extracts and should be further evaluated.

Chapter 4: Conclusion

The aim of this study was to determine the wound healing potential of four crude extracts of *T. sericea*. This was achieved through the investigation of factors that directly affect the wound healing process, such as the determination of bioactive phytochemical classes, antioxidant, antimicrobial, proliferative and migratory activity.

Screening of broad phytochemical classes using TLC indicated the presence of alkaloids, coumarins, flavonoids, glycosides, saponins, sterols and terpenoids in all the extracts with the exception of the HW extract. Phytochemical analysis using UPLC-TOF-MS confirmed the presence of the reported marker compounds of the *Terminalia* genus: punicalagin, sericoside, anolignan B and arjunic acid. The EtOAc extract tested positive for the presence of all four compounds whereas the MeOH extract tested positive for punicalagin, anolignan B and arjunic acid. The HW extract tested positive for punicalagin and sericoside. The HE extract did not test positive for any of the four marker compounds of the genus investigated, which is attributed to the non-polar nature of the extract and the affinity of the compounds for polar solvents.

The MeOH extract displayed significant ($p < 0.0001$) free radical scavenging ability which was more potent than Trolox (PC) in the DPPH assay. The EtOAc extract displayed significant ($p < 0.0001$) free radical scavenging ability more potent than Trolox (PC) in the $ABTS^{\bullet+}$ assay. The HW extract was found to induce the most potent ($p < 0.0001$) protective effects against AAPH induced damage in the CAA assay, displaying a dose dependent decrease in intracellular ROS. The MeOH, EtOAc and HE extracts also exhibited a dose-dependent decrease in intracellular ROS in the CAA assay. The antioxidant activity of *T. sericea* has been attributed to the flavonoid, phenolic acid, lignin and saponin compounds present in the plant. Compounds with high radical-scavenging capacity have been found to facilitate wound healing.[340]

The hexane extracts displayed noteworthy antimicrobial activity against *P. aeruginosa*, *S. epidermidis* and *V. parahaemolyticus* in the broth micro-dilution assay with MIC values equivalent to 1 mg/mL. The antimicrobial activity noted with the MeOH, HW and EtOAc extracts were deemed clinically insignificant as the MIC values exceeded 1 mg/mL. The biofilm inhibition assay revealed that most extracts enhanced the growth of biofilms. This paradoxical effect has been linked to the plant polysaccharide content

which has previously been shown to act as a sugar source that stimulates biofilm growth and gene expression of the exopolysaccharide matrix.[356] Based on the results from the biofilm inhibition assay, it can be concluded that the extracts are not strong anti-biofilm substances and could deter treatment if a mature biofilm has already formed. However, as selected extracts displayed activity against planktonic bacteria, if treatment were to occur before bacterial infection were to set it in, it could assist in killing planktonic bacteria before it progresses to form a biofilm.

A generally low cytotoxic trend was observed with all the extracts, with the exception of the EtOAc and MeOH extracts at higher concentrations (> 32 µg/mL) after a 72 h exposure period. Half-maximal inhibitory concentrations for the HW and HE extracts exceeded the highest concentrations tested after a 24 and 72 h exposure period. These findings suggest that the plant is “safe”, but caution is warranted with the dosing regimen of *T. sericea* extracted with intermediate polarity solvents.

The results from the *in vitro* wound healing assays indicated that the extracts of *T. sericea* enhanced migration but not proliferation. The scratch assay resulted in a significant ($p < 0.05$) enhancement of cell migration in both the fibroblast and endothelial hybrid cell lines. However, no significant increase in cell density was noted with the fibroblast proliferation assay, with the exception of the EtOAc extract at 1 µg/mL ($p \leq 0.05$). The migratory activity has been attributed to phytochemicals such as saponins, tannins, phenolic acids and flavonoids working in an additive or synergistic manner.

The EtOAc and HE extracts were found to be the most promising. The EtOAc extract displayed potent antioxidant, migratory, proliferative and antibacterial activity. This extract was able to significantly ($p < 0.05$) induce cell proliferation at a sub-toxic concentration of 1 µg/mL. The solvent was able to extract all the phytochemicals noted in literature which are reported to be responsible for the respective bioactivities related to wound healing. The HE extract displayed a dose-dependent protective antioxidant effect, noteworthy antibacterial effects and enhanced migratory effects. Apart from these activities, the hexane extract also displayed a low cytotoxic profile, making it an

ideal candidate for further investigation into the development of a wound healing agent.

This study is the first to investigate the protective antioxidant activity in a cellular model, biofilm inhibitory activity and migratory activity of *T. sericea* and is also the first to confirm the presence of punicalagin in the extracts of this specific species. The plant extracts displayed antioxidant and antimicrobial activity indicating that the plant provides protection against oxidative damage and microbial infection in a wound. They also displayed potent migratory properties which relates to their ability accelerate wound closure. The overall wound healing effect of *T. sericea* is proposed to be a combination of the effects that it elicits on the multiple processes that directly affect wound healing. The ethnomedicinal use of *T. sericea* as a wound healing agent was validated.

Chapter 5: Limitations and recommendations

The identification of punicalgin using UPLC-TOF-MS was carried out using a pure standard but the identification of the other bioactive compounds were based on monoisotopic masses obtained from literature. While UPLC-TOF-MS is an accurate technique and confirmed the presence of the compounds in the plant extracts, the intensity of the peaks were low, and in turn it appeared as though the compounds in the extracts were of low abundance. In order to have obtained higher peak intensity pure standards should have been purchased for the other compounds. These pure standards could have also been used to create a calibration curve which would allow for the quantification of the compounds in the extracts.

The SRB assay provides an indication of cell density. Inclusion of a proliferative marker such as Ki67 (a protein present in the proliferative phases of the cell cycle), would have made it possible to distinguish between increased cell density and proliferation.

Since a dose dependent increase in migration was observed as the extract concentration decreased, further investigation into migratory effects at concentrations lower than 1 µg/mL is recommended for future studies. Future studies should also focus on determining the mechanisms whereby migration is activated by the extracts.

While this study investigated the effects of the crude extracts on various factors influencing wound healing, it was not within the scope of the study to determine the effects of the plant on inflammatory factors. This would have provided more clarity on the effects of the plant on the inflammation phase of wound healing.

Another limitation of this study was the use of a two dimensional model, while this model is well established and extensively used, it lacks the complexity of a wound microenvironment. A suggestion for future studies would be the use of a three dimensional *in vitro* model which can accommodate multiple cell types as well as structural elements such as collagen and fibrin. Following the evaluation of the extracts in a 3D model an animal model, such as pig model could be used to further evaluate the wound healing effects. An animal model would take into account factors such as the immune component of the wound healing process in addition to other factors such

as activity of the extracts in the presence of serum, tissue cellular structure and inflammatory and proliferative markers.

For future studies, it is recommended that the most active compounds be isolated and biological activity be assessed, which will hopefully lead to the development of a product that could be employed as a wound healing agent.

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Appendix I: 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 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
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Faculty of Health Sciences Research Ethics Committee

17/10/2014

Approval Certificate
New Application

Ethics Reference No.: 387/2014

Title: Wound Healing potential of Terminalia sericea

Dear Miss Hafiza Parkar

The **New Application** as supported by documents specified in your cover letter for your research received on the 25/08/2014, was approved by the Faculty of Health Sciences Research Ethics Committee on the 17/10/2014.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year.
- Please remember to use your protocol number (**387/2014**) 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.
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 2004 (Department of Health).

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*** Kindly collect your original signed approval certificate from our offices, Faculty of Health Sciences, Research Ethics Committee, H W Snyman South Building, Room 2.33 / 2.34.*

Appendix II: List of reagents

AAPH

AAPH powder was obtained from Sigma-Aldrich (St. Louis, United States of America) and stored at 4ºC till use. Prior to use a 5 mM working solution was prepared in DMEM which was diluted in well to final concentration of 2.5 mM.

ABTS

2,2'-azinobis-(3-ethyl benzothiazoline 6-sulfonic acid) (ABTS) and potassium peroxidisulfate was obtained from Sigma-Aldrich (St. Louis, United States of America) in powder form. A 7.46 mM of ABTS and 2.5 mM of potassium peroxidisulfate solution was prepared by dissolving 38.4 mg ABTS and 6.62 mg potassium peroxidisulfate in 10 mL distilled water and incubating for 16 h at 4ºC.

Acetic acid

Acetic acid was obtained from Saarchem (Pty) Ltd (Krugersdorp, South Africa) and Sigma-Aldrich (Pty) Ltd (Kempton Park, South Africa) as a liquid and stored at room temperature until use. Acetic acid (10 mL) was diluted with 990 mL of dH_2O and stored at room temperature in a clear glass container until use.

Acetone, ammonium hydroxide, chloroform, ethanol, ethyl acetate sulphuric acid and toluene

Acetone, ammonium hydroxide, chloroform, ethanol, ethyl acetate, methanol, sulphuric acid and toluene were obtained from Merck (Pty) Ltd (Modderfontein, South Africa) as liquids, were stored at room temperature and used undiluted.

Crystal Violet

Crystal Violet was procured from Merck (Pty) Ltd (Modderfontein, South Africa) in a powdered form. A 0.1% solution was prepared in distilled water and stored in a clear glass container at room temperature till use.

DCFDA

DCFDA in was obtained from Sigma-Aldrich (St. Louis, United States of America) in a powdered form. A 1 mg/mL stock solution (2 mM) was prepared in methanol and stored at - 20ºC. The stock solution was diluted in PBS to 10 µM prior to use.

DMEM

DMEM was obtained from Sigma-Aldrich (St. Louis, United States of America) in powder form. A 1.04% solution was prepared in autoclaved, ultra-pure, pyrogen-free, deionized water and adjusted to pH 7.4 using sodium hydrogen carbonate obtained from Merck Chemicals (Darmstadt, Germany) in powder form. The solution was filtered three times using *in vacuo* filtration (Sartorius, 0.22 µm) and supplemented with 1% penicillin/streptomycin and stored at 4ºC until used.

DMSO

DMSO was attained from Sigma-Aldrich (St. Louis, United States of America) and used undiluted.

DPPH

DPPH was obtained from Sigma-Aldrich (St. Louis, United States of America) in powder form. A stock solution of 600 µM was prepared by dissolving 11.8 mg DPPH in 50ml methanol and sonicating for 20 min. A working solution of 120 µM was prepared by diluting 20 ml stock solution with 80 ml methanol and sonicating for 20 min.

Dragendorff's Reagent

Bismuth nitrate and potassium iodide were obtained from Merck Chemicals (Darmstadt, Germany) in a powdered form. Two solutions were prepared: A) 1.7% bismuth nitrate in 20% aqueous acetic acid, and B) 13% potassium iodide solution in 30% aqueous acetic acid. Solutions A and B were mixed in 4:1 ratio just prior to use.

Hexane

Hexane was procured from Sigma-Aldrich (Kempton Park, South Africa) as a liquid, used undiluted and stored at room temperature till use.

FCS

FCS was acquired from The Scientific Group (Gauteng, South Africa) and heat-inactivated through heating at 56ºC for 45 min. The solution was stored at -20ºC till use.

Follin-Ciocalteau Reagent

Folin-Ciocalteau reagent (2 N) was obtained from Sigma-Aldrich (St.Louis, United States of America) in liquid form and used undiluted.

Methanolic Potassium Hydroxide (5%)

Potassium hydroxide was obtained in a powdered form from Associated Chemical Enterprises (Johannesburg, South Africa). 5 g of was dissolved in 100 mL of methanol.

Mueller-Hinton Agar

Meuller-Hinton agar was procured from Davies Diagnostics (Randburg, South Africa) in a powdered form. Agar was prepared by dissolving 18 mg of powder to 500 mL of dH_2O and heated to 100ºC. The agar was autoclaved at 121ºC for 15 min and allowed to cool to 50-55ºC prior to use.

Meuller-Hinton Broth

Meuller-Hinton broth was procured from Davies Diagnostics (Randburg, South Africa) in a powdered form. Broth was prepared by dissolving 12 mg of powder to 500 mL of distilled was and autoclaved at 121ºC for 15 min. The broth was stored in a clear glass container at 4ºC until use.

Penicillin/Streptomycin

A penicillin (10 000 U) and streptomycin (10 000 µg/mL) solution was acquired from BioWhittaker (Walkersville, USA). The solution was added to culture medium to a final concentration of 1%.

PDGF

PDGF powder was purchased from Sigma-Aldrich (St. Louis, United States of America). A stock solution of 1000 ng was prepared in PBS supplemented with 0.1% BSA obtained from Santa Cruz Biotechnology (California, USA), aliquoted and stored at -80ºC until use. The stock solution was used to prepare a 10 ng working solution in DMEM which was diluted in well to a final concentration of 2 ng.

Phosphate Buffered Saline

BBL™ FTA hemagglutination buffer was obtained from BD Scientific (Paris, France) in powder form. A 0.9% solution was prepared in distilled water and stored at 4ºC.

Phosphoric Acid (85%) in dH_2O

Phosphoric acid was obtained from Merck Chemicals (Darmstadt, Germany) as a liquid. A solution was prepared by adding 8.5 mL of phosphoric acid to 1.5 mL of dH_2O prior to use.

P-INT

p-Iodonitrotetrazolium powder was procured from Sigma-Aldrich (St. Louis, United States of America). A 3% working solution was prepared in PBS prior to use.

Saponin

Saponin was obtained from Sigma-Aldrich (St. Louis, United States of America) in powder form. A 1% solution was prepared in DMEM and stored at 4ºC.

Sodium nitrate (3%), aluminium trichloride (1%) and sodium hydroxide (2%)

Sodium nitrate and sodium hydroxide were obtained from Merck Chemicals (Darmstadt, Germany) in a powdered form. Aluminium trichloride powder was obtained from Sigma-Aldrich (St. Louis, USA). The solution was prepared by dissolving 0.45 g of sodium nitrate, 0.15 g of aluminium trichloride and 0.3 g of sodium hydroxide powder in 15 mL of dH_2O.

Sulphorhodamine B

SRB was obtained from Sigma-Aldrich (Kempton Park, South Africa) in powdered form. SRB powder (285 mg) was dissolved in 500 mL of 1% acetic acid and stored in 500 mL foil covered plastic container until used.

Trichloroacetic acid

Trichloroacetic acid was obtained from Merck Chemicals (Darmstadt, Germany) in crystalline form. A 50% solution was prepared in dH_2O and stored in a plastic container at 4ºC until use.

Tris-Base Solution

Tris-base was procured from Research Organics Inc. (Cleveland, United States of America) in powdered form. Tris base was prepared by dissolving 600 mg of the powder in 500 mL of dH_2O. The solution was adjusted to pH 10.5 and stored in a 500 mL clear plastic container at room temperature till use.

Trypan Blue

Trypan Blue was obtained from Sigma-Aldrich (St. Louis, United States of America) in powder form. A 0.1% solution was prepared in distilled water and stored in a foil covered plastic tube at room temperature until use.

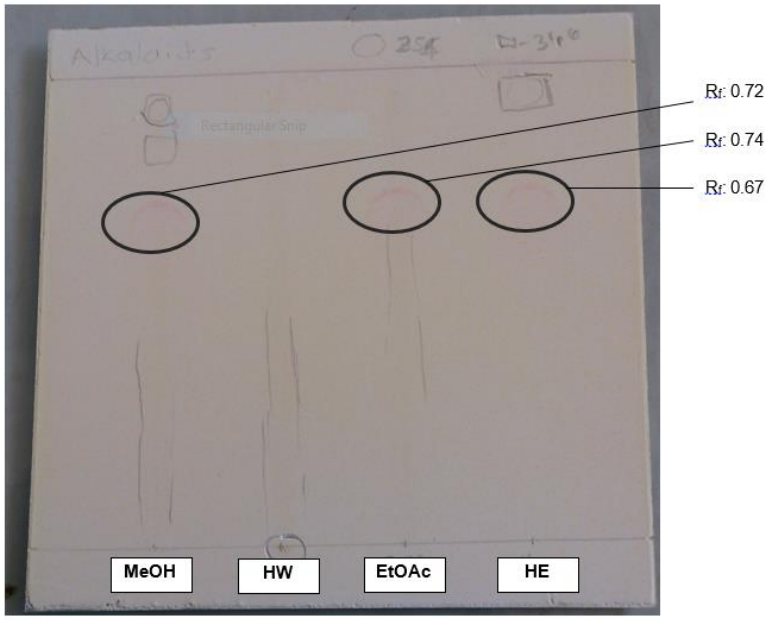
Vanillin (1%) Sulphuric acid (5%)

Vanillin powder was obtained from Sigma-Aldrich (St. Louis, United States of America). Prior to use 0.1 g of vanillin powder was dissolved in 0.5 mL of sulphuric acid, this solution was further dissolved in 9.4 mL of dH_2O.

Appendix III: Thin layer chromatography

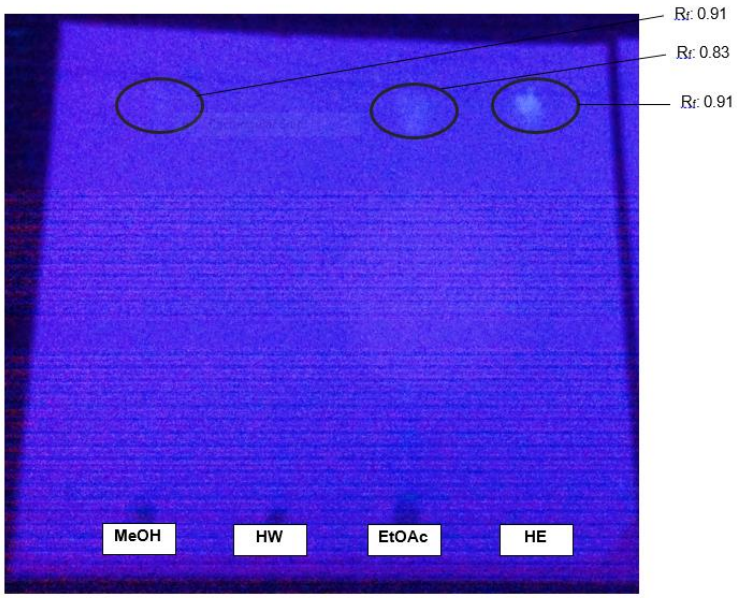
TLC images indicating the presence of A) alkaloids, B) coumarins, C) flavonoids, D) glycosides, E) phenolic acids, F) saponins, H) sterols and G) terpenoids.

A)



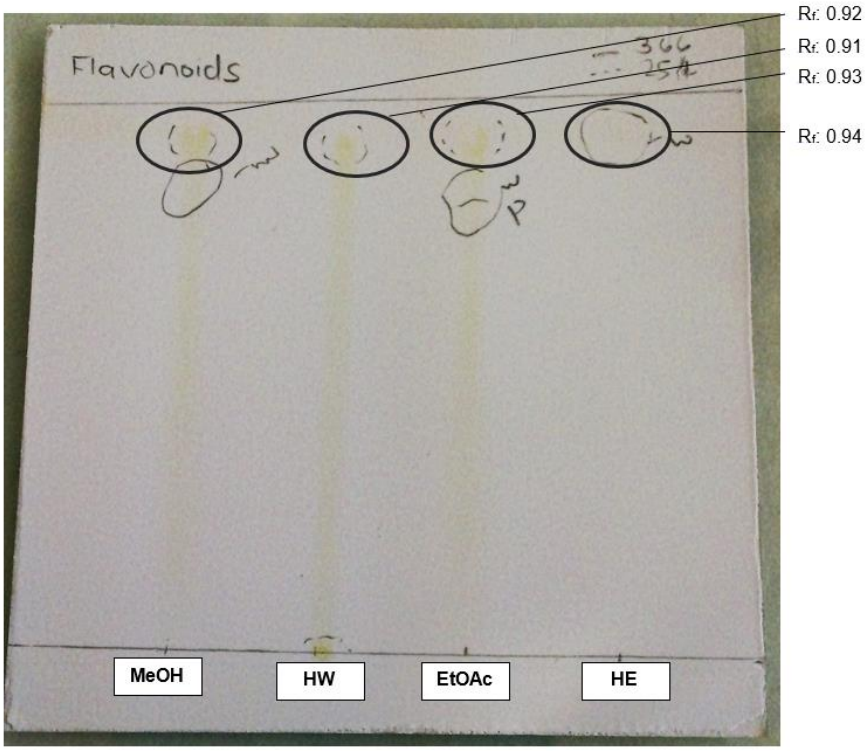
TLC chromatogram of alkaloids (orange bands) present in *T. sericea* crude extracts after development and spraying with Dragendorff's reagent.

B)



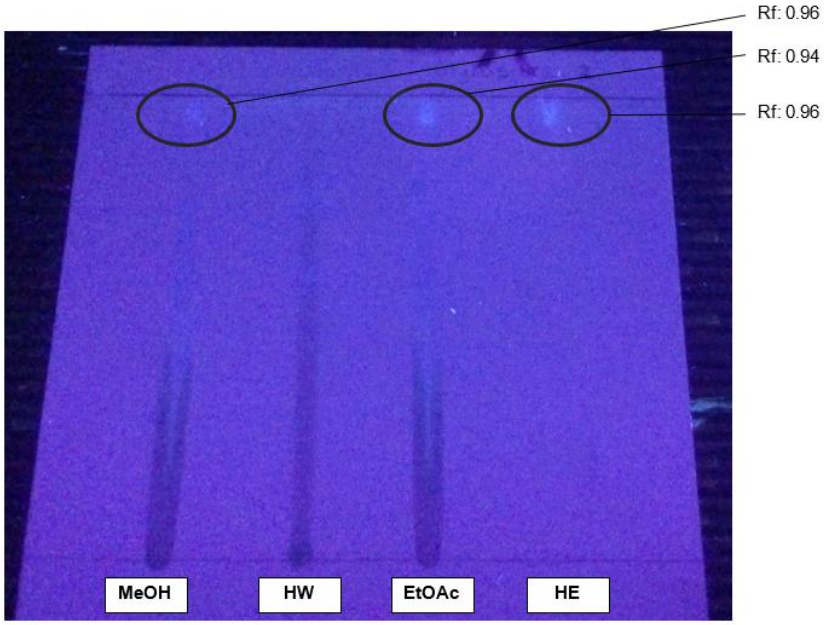
TLC chromatogram of coumarins (blue spots) present in *T. sericea* crude extracts visualised under UV 366 nm.

C)



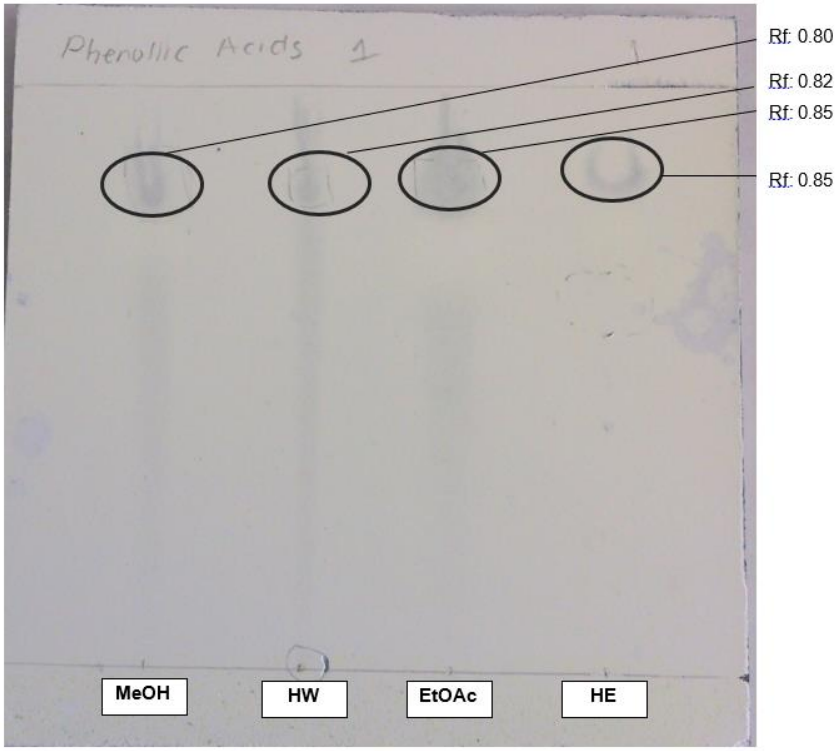
TLC chromatogram of flavonoids (yellow spots) present in *T. sericea* crude extracts after spraying with 3% sodium nitrate:1% aluminum trichloride:0.5M sodium hydroxide.

D)



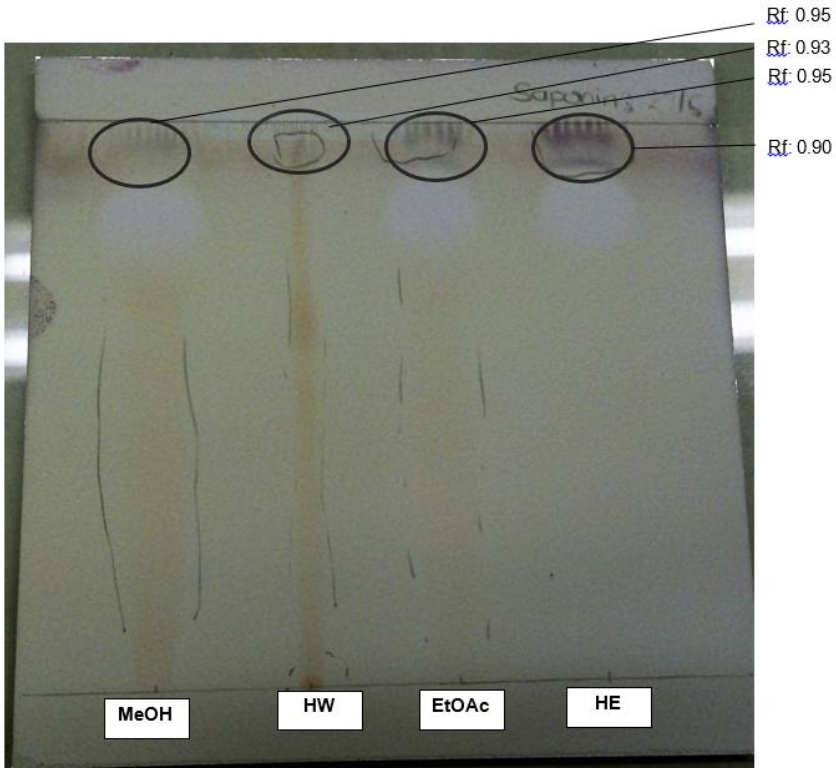
TLC chromatogram of glycosides (white spots) present in *T. sericea* crude extracts visualised under UV 366 nm.

E)



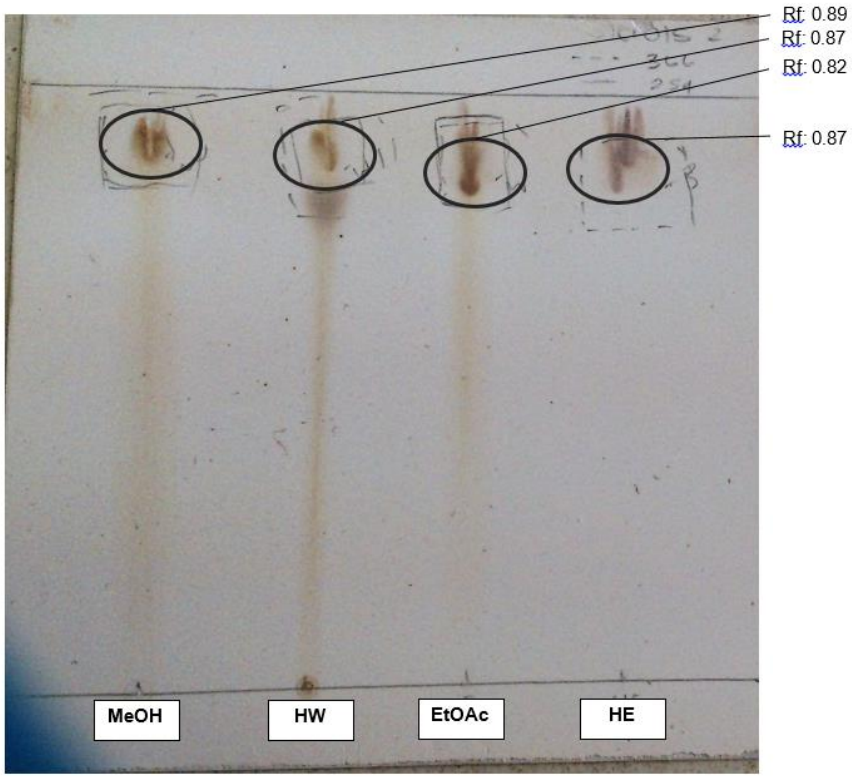
TLC chromatogram of phenolic acids (grey spots) present in *T. sericea* crude extracts after spraying with Folin-Ciocalteau reagent.

F)



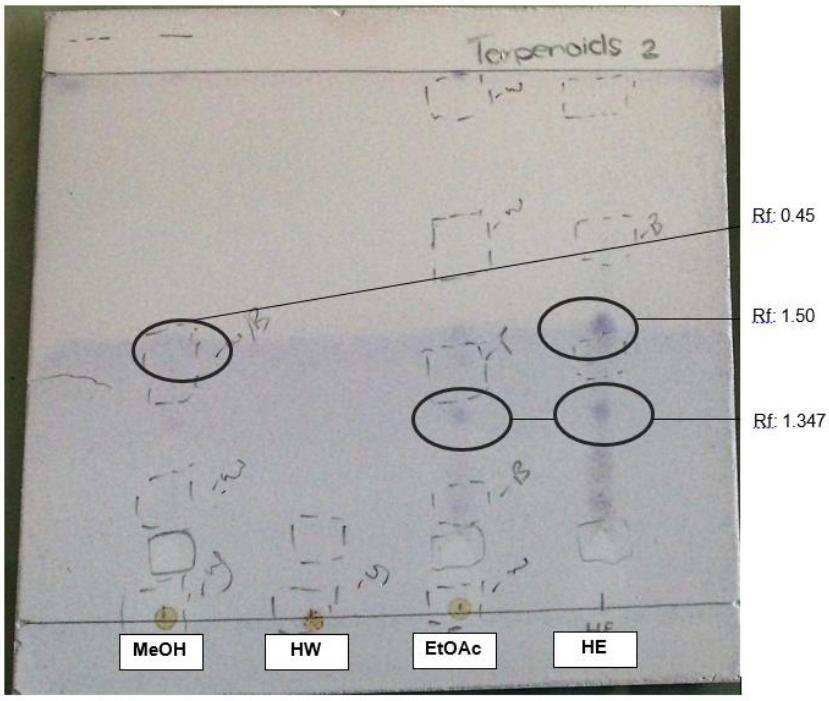
TLC chromatogram of saponins (purple spots) present in *T. sericea* crude extracts after spraying with 1% vanillin in a 5% sulphuric acid solution.

G)



TLC chromatogram of sterols (red-brown spots) present in *T. sericea* crude extracts after spraying with 85% phosphoric acid in ddH_2O.

H)

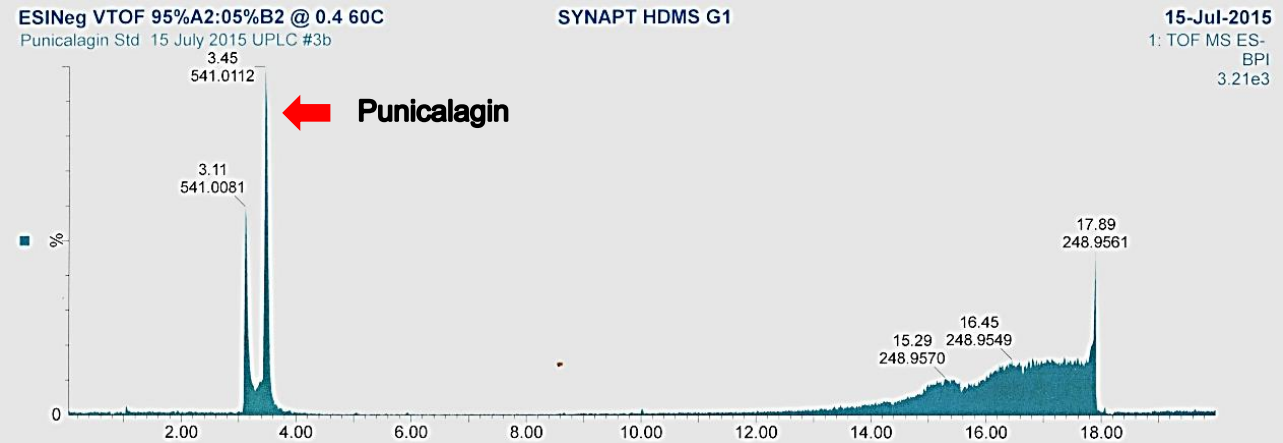


TLC chromatogram of terpenoids (blue/grey spots) present in *T. sericea* crude extracts after spraying with 1% vanillin in a 5% sulphuric acid solution.

Appendix IV: UPLC-TOF-MS chromatography

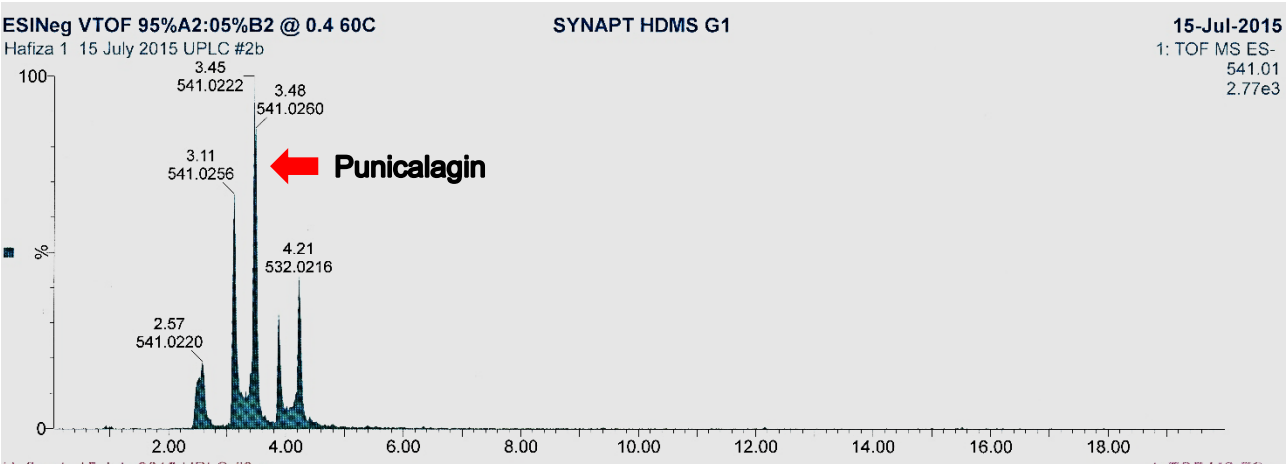
Chromatograms indicating the presence of A) punicalagin, B) sericoside, C) anolignan B and D) arjunic acid in the extracts of *T. sericea.*

A)



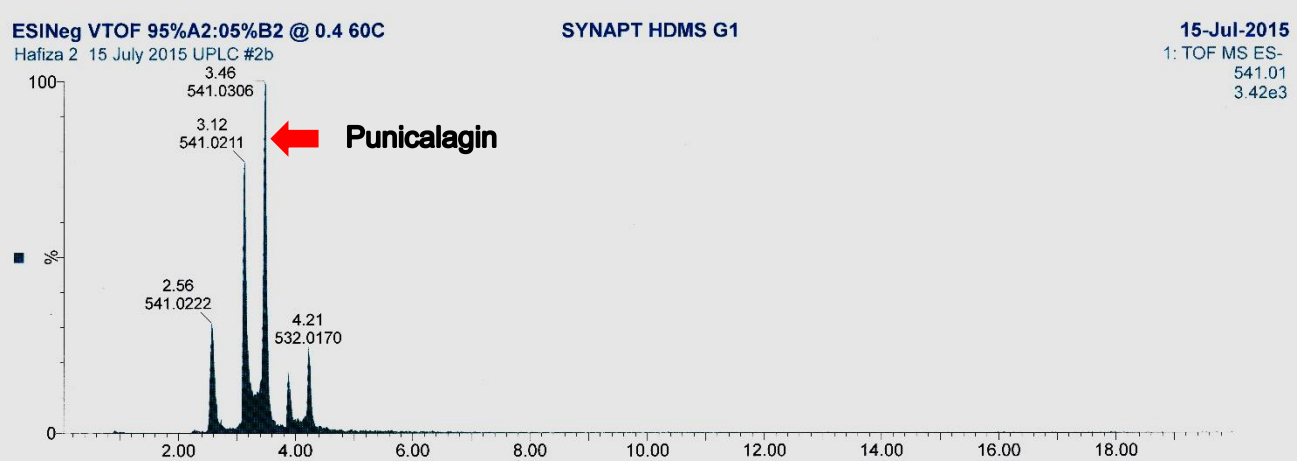
UPLC-TOF-MS chromatogram of punicalagin standard in elecrospray negative (ESI -) mode.

B)



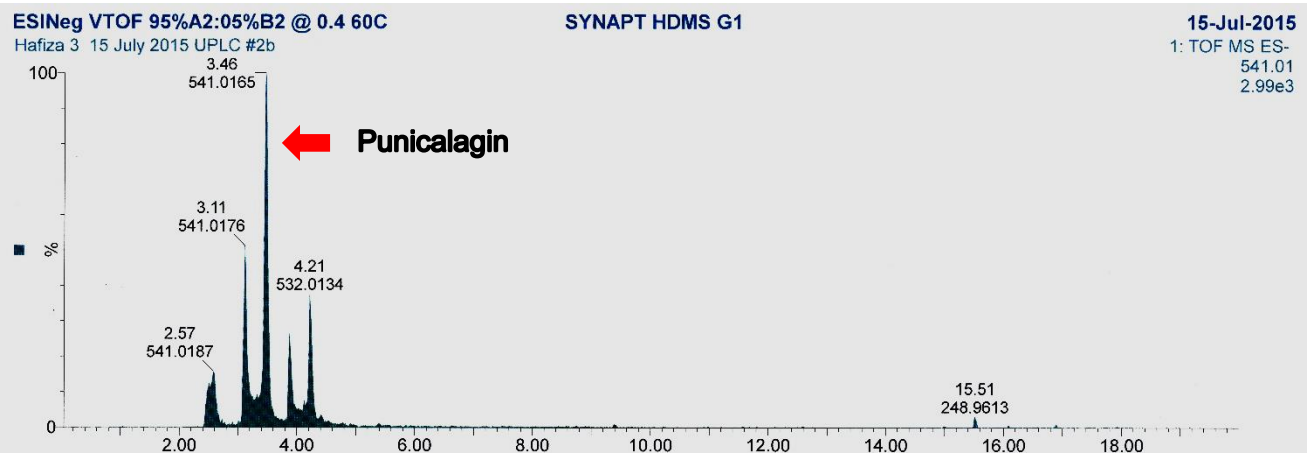
UPLC-TOF-MS chromatogram of MeOH extract of *T. sericea* containing punicalagin in ESI (-) mode.

C)



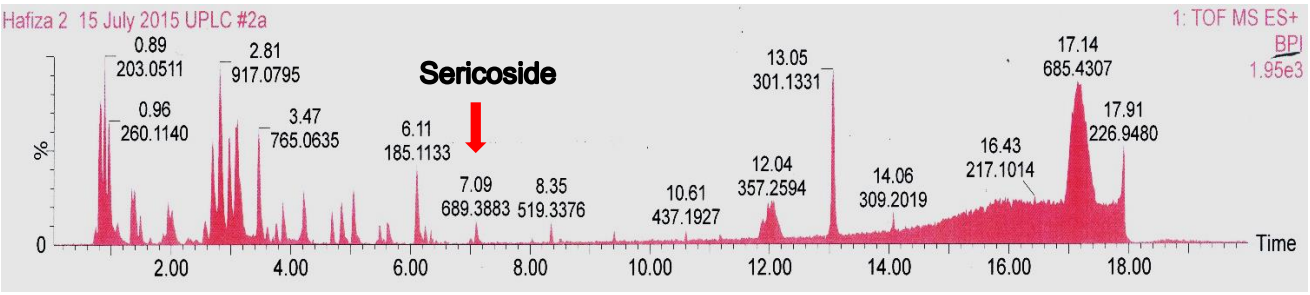
UPLC-TOF-MS chromatogram of HW extract of *T. sericea* containing punicalagin in ESI (-) mode.

D)



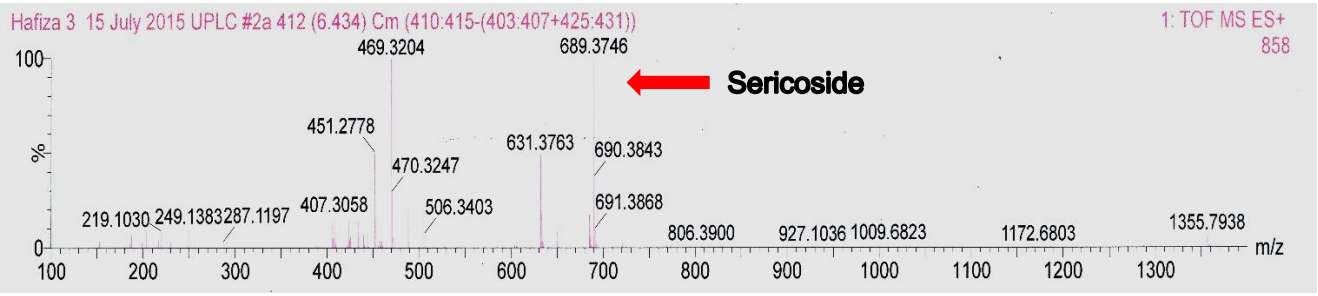
UPLC-TOF-MS chromatogram of EtOAc extract of *T. sericea* containing punicalagin in ESI (-) mode.

E)



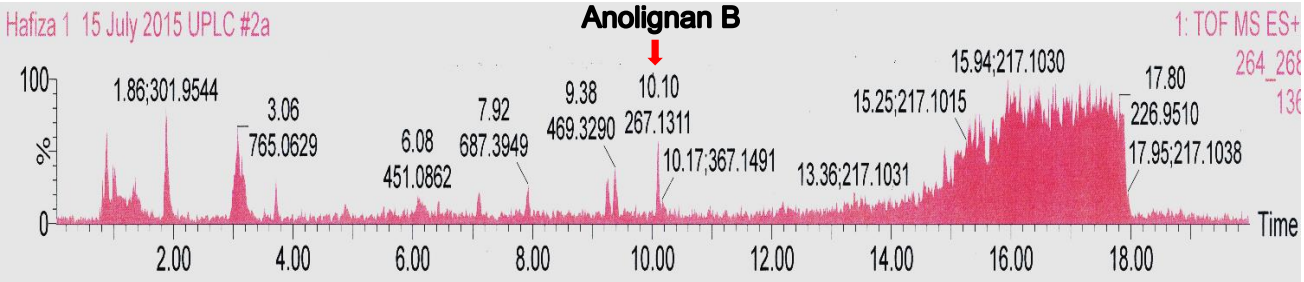
UPLC-TOF-MS chromatogram of HW extract of *T. sericea* containing sericoside in ESI (+) mode.

F)



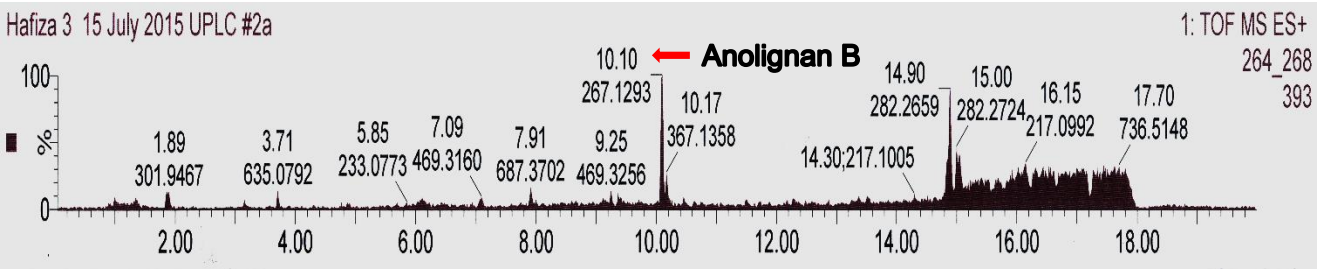
UPLC-TOF-MS fragmentation pattern of HW extract of *T. sericea* containing sericoside in ESI (+) mode.

G)



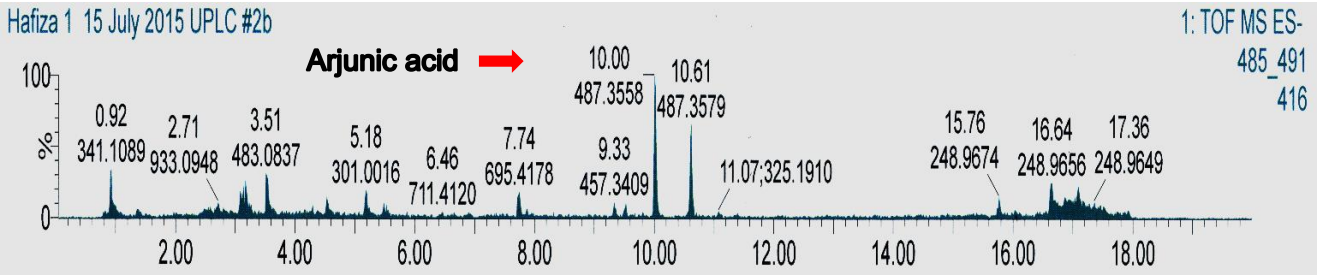
UPLC-TOF-MS chromatogram of MeOH extract of *T. sericea* containing anolignan B in ESI (+) mode.

H)



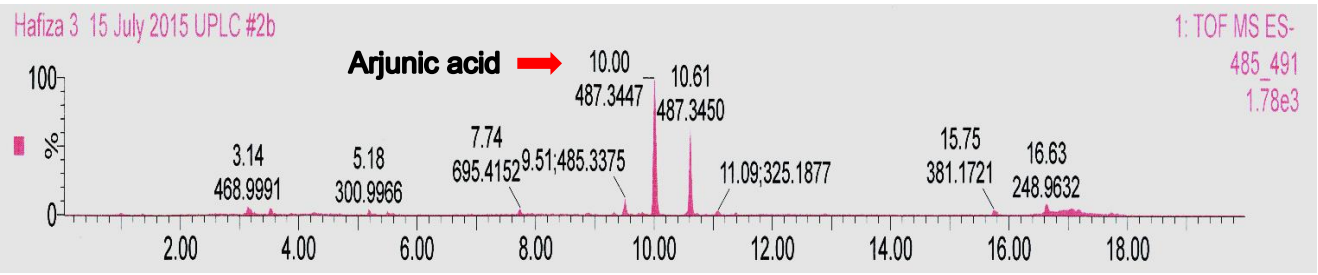
UPLC-TOF-MS chromatogram of EtOAc extract of *T. sericea* containing anolignan B in ESI (+) mode.

I)



UPLC-TOF-MS chromatogram of EtOAc extract of *T. sericea* containing arjunic acid in ESI (-) mode.

J)



UPLC-TOF-MS chromatogram of EtOAc extract of *T. sericea* containing arjunic acid in ESI (-) mode.