Cape Peninsula
University of Technology

AN *IN-VITRO* EVALUATION OF THE WOUND HEALING PROPERTIES OF *COTYLEDON ORBICULATA* EXTRACTS AND ITS SILVER NANOPARTICLES

By

CAROLINE TYAVAMBIZA

Thesis submitted in fulfilment of the requirements for the degree

Doctor of Philosophy: Biomedical Sciences

in the

Faculty of Health and Wellness Sciences

at the

Cape Peninsula University of Technology

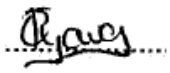
Supervisor: Dr S. Meyer

Co-supervisor: Prof. M. Meyer

Bellville, 2022

DECLARATION

I, Caroline Tyavambiza, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.



29/10/2022

Signed **Date**

ABSTRACT

Chronic wounds are a silent epidemic affecting a large segment of the world's population. They are associated with severe healthcare and socio-economic burdens, and are a great financial burden on healthcare institutions worldwide. Medicinal plants have been used traditionally as a source of medicine. To date, medicinal plants continue to play a significant role in the treatment of wounds. The synthesis of silver nanoparticles using chemicals derived from biological systems, particularly plants led to the discovery of nanoparticles with useful bioactivities. In many instances, plant derived silver nanoparticles have been shown to have higher activity than the plant extract itself. The plant, *Cotyledon orbiculata*, a medicinal plant from South Africa has been used traditionally for the treatment of skin wounds. Solvent extracts of the plant have been shown to have good antimicrobial, antioxidant and anti-inflammatory activities. Silver nanoparticles (*Cotyledon*-AgNPs) synthesized from the water extract of *C. orbiculata* were also reported to exhibit good antimicrobial and anti-inflammatory activities however, their antioxidant and wound healing properties, as well as the cytotoxicity have not been determined. This study therefore aimed to investigate these properties of *C. orbiculata* extract and *Cotyledon*-AgNPs.

The antioxidant activity of *C. orbiculata* extract and *Cotyledon*-AgNPs was determined using the Ferric Reducing Antioxidant Power and the 2'-Azino-Bis-3-Ethylbenzotiazolin-6- Sulfonic Acid assays. The wound healing activity of *C. orbiculata* extract and *Cotyledon*-AgNPs was investigated using the cell growth and the wound healing scratch assay. Gene expression studies using real time qPCR were also performed to underpin the molecular mechanisms of wound healing exerted by *C. orbiculata* extract and *Cotyledon*-AgNPs treatments. The cytotoxic effects of the *Cotyledon*-AgNPs on non-cancerous skin fibroblast cells (KMST-6) were also investigated using in vitro bioassays and real time qPCR. Several in vitro assays, specifically the Mitochondrial Membrane Potential assay, APOPercentage^{TM} assay, and the oxidative stress assay were used to study the cytotoxicity of the treatments.

Both the *C. orbiculata* extract and *Cotyledon*-AgNPs showed the presence of polyphenols, flavanols, tannins and flavonols. They also exhibited some antioxidant activity however the extract showed more reducing activity than the *Cotyledon*-AgNPs. Some of these

phytochemicals have been shown to possess antimicrobial and anti-inflammatory activities. At low concentrations, *Cotyledon*-AgNPs promoted cell growth and cell migration. Gene expression studies showed that the *Cotyledon*-AgNPs promoted wound healing by upregulating genes involved in cell proliferation, migration and growth while downregulating pro-inflammatory genes. This confirmed that *C. orbiculata* and *Cotyledon*-AgNPs are potentially good wound healing agents. The toxic effects of the *Cotyledon*-AgNPs to non-cancerous skin fibroblasts were determined using in vitro assays. The *Cotyledon*-AgNPs did not show any significant mitochondrial or cellular damage; instead it exhibited signs of cell proliferation. Gene expression studies showed the upregulation of genes involved in fatty acid and mitochondrial energy metabolism, steatosis, cholestasis, and apoptosis. The study shows that *Cotyledon*-AgNPs are not toxic to skin fibroblasts at the concentration that promote cell growth and wound healing.

This study provides scientific evidence which lends credibility to the traditional use of *C. orbiculata* for the treatment of skin wounds. It also shows that *C. orbiculata* derived silver nanoparticles are non-toxic potent wound healing agents. In the future these nanoparticles for can be used in the development of novel wound healing treatments.

ACKNOWLEDGEMENTS

- I would like to thank God for being with me throughout this journey. It had its ups and downs but God saw me through it all. I am grateful.
- To my supervisors, Dr Samantha Meyer and Prof Mervin Meyer, thank you for moulding me into the researcher I am today. Thank you for the mentorship, encouragement, teachings and support you gave throughout my postgraduate studies.
- I would like to thank the Vice Chancellors Prestigious Awards programme, National Research Foundation and DSI/Mintek Nanotechnology Innovation Centre for the financial support.
- I would also want to thank my colleagues from the CPUT Phytotherapy research group and UWC NIC group for working well together.
- I wish to thank my former colleagues who are now Drs for their assistance in the lab, encouragement and motivation. Thank you Dr Phumuzile Dube, Dr Mediline Goboza, Dr Jumoke Aboyewa, Dr Nicole Sibuyi and Dr Dorcas Wusu.
- A very special thanks to my family: my mom and dad, thank you for your sacrificial love and support, thank you for believing in me. I would also like to thank my cousins, nephews, nieces, aunties, uncles and my precious in-laws. I appreciate you all.
- I sincerely appreciate my husband (Tatenda Runganga) for the unfailing support and encouragement you always give. Thank you for being my first “editor”…I will always love you.

DEDICATION

To my brother

It was difficult completing this journey without you.
It was as if a part of me was also gone.
Remembering your love and support, gave me the strength to successfully finish this journey, I hope you are proud of me.

Continue to rest in peace my STANY, I will always love you.

LIST OF PUBLICATIONS

Published:

Tyavambiza, C., Dube, P., Goboza, M., Meyer, S., Madiehe, A.M., Meyer, M. 2021. Wound Healing Activities and Potential of Selected African Medicinal Plants and Their Synthesized Biogenic Nanoparticles. *Plants*. 10 (2635): 1-14.

Tyavambiza, C., Meyer, M., Meyer, S. 2022. Cellular and molecular events of wound healing and the potential of silver based nanoformulations as wound healing agents. *Bioengineering*. 9 (712): 1-17.

Tyavambiza, C., Meyer, M., Wusu, D.A., Madiehe, A.M., Meyer, S. 2022. The antioxidant and in vitro wound healing activity of *Cotyledon orbiculata* aqueous extract and the synthesized biogenic silver nanoparticles. *International Journal of Molecular Sciences. 23* (16094): 2-20.

Manuscripts ready for submission:

Tyavambiza, C., Meyer, M., Meyer, S. 2022. The cytotoxicity of *Cotyledon orbiculata* aqueous extract and the biogenic silver nanoparticles derived from the extract.

LIST OF CONFERENCES AND PRESENTATIONS

1. **Caroline Tyavambiza***, Mervin Meyer and Samantha Meyer. The Antimicrobial and Anti-Inflammatory Effects of Silver Nanoparticles Synthesized from *Cotyledon orbiculata* Aqueous Extract. **South African Society for Biochemistry and Molecular Biology**. 23-26 January 2022. South Africa, Online conference. Poster Presentation. (* - presenter)

2. **Caroline Tyavambiza***, Mervin Meyer and Samantha Meyer. The Antimicrobial and Anti-Inflammatory Effects of Silver Nanoparticles Synthesized from *Cotyledon orbiculata*. **11th Annual BRIP Symposium**. 18-19 October 2021. South Africa, Online conference. Oral Presentation.

3. **Caroline Tyavambiza***, Mervin Meyer and Samantha Meyer. The Antimicrobial and Anti-inflammatory effects of metallic nanoparticles synthesized from indigenous South African plants. **Nano4Youth Seminar 2021**. 28 June 2021. South Africa, Online conference. Oral Presentation.

4. **Caroline Tyavambiza***, Mervin Meyer and Samantha Meyer. The antimicrobial activity of *Cotyledon orbiculata* against antimicrobial resistant microorganisms. **World Antimicrobial Awareness Week 2020**. 19-20 November 2020. South Africa, Online conference. Oral Presentation.

5. Mervin Meyer*, Abdulrahman Elbagory, Phumuzile Dube, **Caroline Tyavambiza**, Ahmed Hussein, Samantha Meyer, Abram Madiehe and Martin Onani. The antimicrobial and immune modulatory effects of bio-inspired nanoparticles produced from indigenous flora of South Africa. **10th International Conference of the African Materials Research Society**, 10-13 December 2019. Arusha, Tanzania. Plenary Talk.

6. Mervin Meyer*, Abdulrahman Elbagory, Phumuzile Dube, **Caroline Tyavambiza**, Ahmed Hussein, Samantha Meyer, Abram Madiehe and Martin Onani. The antimicrobial and immune modulatory effects of bio-inspired nanoparticles produced from indigenous flora of South Africa. **Nanotechnology and Artificial Intelligence.** 16-17 October 2019. Rome, Italy. Oral presentation.

7. Samantha Meyer*, **Caroline Tyavambiza**, Mervin Meyer. The biomedical application of biogenic silver nanoparticles produced from *Cotyledon orbiculata*. **3rd Edition of Global conference on Pharmaceutics and Drug Delivery Systems**. 24-26 June 2019. Paris, France. Poster Presentation.

8. **Caroline Tyavambiza***, Mervin Meyer and Samantha Meyer. *Cotyledon orbiculata*: a study of its relevance for skin infections. **6th U6 International Conference**. 4-6 September 2018. South Africa, Cape Town. Oral presentation.

Table of Contents

LIST OF ABBREVIATIONS

AgNPs	Silver nanoparticles
AIDS	Acquired Immune Deficiency Syndrome
ATP	Adenosine triphosphate
DLS	Dynamic Light Scattering
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DPPH	Di-phenyl-picrylhydrazyl
ECM	Extracellular Matrix
EDX	Energy-dispersive X-ray spectroscopy
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FRAP	Ferric reducing antioxidant power
HIV	Human Immunodeficiency Virus
HRTEM	High-Resolution Transmission Electron Microscopy
IFN-γ	Interferon-gamma
IL	Interleukin
MMP	Matrix metalloproteinase
ORAC	Oxygen radical antioxidant capacity
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction (PCR
PDGF	Platelet-derived Growth Factor
PDI	Poly Dispersity Index
ROS	Reactive oxygen Specie

RPMI	Roswell Park Memorial Institute
SAED	Selected area electron diffraction
TGF	Transforming Growth Factor
TMRE	Tetramethyl rhodamine ethyl ester
TNF-α	Tumor Necrosis Factor-α
UV-Vis	Ultraviolet visible
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
WST1	Water Soluble Tetrazolium Salts

CLARIFICATION OF TERMS

Angiogenesis: the formation of new blood vessels.

Antioxidants: substances which remove or inhibit potentially damaging oxidizing agents

Apoptosis: a programmed cell death or a mechanism that allows cells to self-destruct.

Chemotaxis: the movement of leukocytes from the blood vessel into a damaged area in response to chemicals from the damaged tissue.

***Cotyledon orbiculata*:** South African medicinal plant traditionally used to treat a variety of wound and skin infections.

Debridement: the removal of necrotic tissue from an affected area in-order to promote wound healing.

Genes: small sections of DNA on a chromosome which code for a particular protein or for a particular function.

Medicinal plants: plants containing properties or compounds that can be used for therapeutic purposes.

Nanotechnology: a field of science dealing with the synthesis, development and use of materials at the nanometer scale.

Phytochemicals: biologically active chemical compounds occurring naturally in plants.

Proliferation: rapid multiplication or increase in the amount of a certain compound.

Silver: a valuable metallic element found in nature that is used for making jewellery and ornaments.

Transcription: the process of gene expression in which a gene's DNA sequence is copied to make an RNA molecule.

Vasodilation: the widening of blood vessels which decreases blood pressure.

Wound: an injury to a living tissue leading to the disruption of its normal structure and function.

Wound healing: a complex physiological process which aims to restore the anatomical structure and function of an injured tissue

LIST OF FIGURES

Figure 4. A shows the apoptotic effects of *Cotyledon*-AgNPs and water extract on KMST-6 cells. **B** shows the flow cytometry plot showing apoptotic and live cells.

Figure 5. Clustergram and fold changes of DEGs.

Figure 6. Protein networks showing the interactions between the DEGs. These networks were determined using the STRING database.

LIST OF TABLES

PREFACE

This thesis is written in an article-based format and consists of 5 chapters. Chapter 1 includes an introduction, aims, objectives and the research rationale. Chapter 2 is the literature review which gives a description of wounds, wound types and the wound healing process. It also provides information on the properties of good wound healing agents including medicinal plants, and their derived silver nanoparticles. Chapter 2 is divided into 2 review articles. One is published and the other has been submitted to a journal for publication. Chapters 3 and 4 are research article manuscripts ready for submission to journals for publication. These chapters focus on the bioactivities of *Cotyledon orbiculata* silver nanoparticles, which was the aim of this study. Chapter 5 is the general discussion; it gives a summary of the discussions and conclusions of the entire thesis. As chapters 2, 3 and 4 focuses on different aspects of the research project they have separate abstracts, introductions, methods, results, discussions, conclusions and references. The published and submitted manuscripts are in the respective journal format.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Nanotechnology is the manipulation of matter on an atomic, molecular and supramolecular scale; with at least one dimension of the material sized from 1 to 100 nm. It has promised significant scientific advancement in many sectors including medicine, electronics, physics, material science, manufacturing and agriculture (Aboyewa et al., 2021). Nanomaterials were primarily synthesized using chemical and physical methods; however, these methods are costly, and they involve the use of toxic chemicals and solvents which are unsafe for the environment (Iravani et al., 2014). In order to reduce the toxicity of nanomaterials, researchers and scientists have turned to green nanotechnology. This is a field of nanotechnology which aims to synthesize bio-friendly nanomaterials using methods and materials (such as plants and microorganisms) that are safer for the environment (Elbagory et al., 2016). Various types of nanomaterials such as metallic nanoparticles, nanorods, nanostars, nanospheres and nanorods have been identified (Siddique & Chow, 2020). Among the many metallic nanoparticles (gold, silver, zinc, platinum) reported in the literature, silver nanoparticles (AgNPs) have become popular due to their good antimicrobial properties (Tyavambiza, Elbagory, et al., 2021). They have been shown to exert good antimicrobial activity even against drug resistant microorganisms such as methicillin-resistant *Staphylococcus aureus* MRSA, *P. aeruginosa* and ampicillin-resistant *Escherichia coli* (Rai et al., 2012). Due to their fundamental therapeutic properties, AgNPs are being incorporated in wound healing as they are anticipated to aid wound healing while controlling and resolving the complication of infections in the wounds.

Wounds are a global health burden to many individuals and to healthcare facilities worldwide. In fact, chronic wounds are a silent epidemic affecting a large fraction of the world's population. It is estimated that 6.5 million people suffer from chronic wounds worldwide (Dreifke et al., 2015). They have a significant impact on the health, social, economic and emotional lifestyle of patients and their families. Chronic wounds can cause severe physical disability and premature death, thereby increasing the mortality and morbidity rate (Järbrink et al., 2017). The effective treatment of wounds is therefore of utmost importance. Unfortunately, the care, management and treatment of chronic wounds is difficult and very costly. In most cases advanced therapies such as growth factors, extracellular matrices and engineered skin are needed, however these are highly expensive and can cause some unwanted side effects (Frykberg & Banks, 2015). Wounds therefore pose a great financial burden on patients and healthcare institutions in both developed and developing countries. In many countries including South Africa, the prevalence of chronic

wounds is increasing due to the increase in conditions impeding wound healing, such as infections, diabetes, and obesity (Jones et al., 2018). Infections exaggerate the challenges of chronic wound treatment, the ability of highly infectious microbes to develop resistance to conventional treatment has made it difficult to eradicate and control infections in chronic wounds (Guo & Dipietro, 2010). The discovery of effective, affordable and accessible treatments for chronic wounds is therefore important.

A good wound healing agent is one that has antimicrobial, antioxidant, anti-inflammatory and growth promoting properties. Ionic silver and AgNPs have been reported to possess many of these properties. In recent years, a number of silver and AgNP-based treatments have been discovered and produced. These include the widely used Acticoat, silverlon, Aquacel Ag and Meliplex Ag, which are known to promote wound healing whilst reducing pain and infection at the wound site (Tyavambiza, Dube, et al., 2021; Khansa et al., 2019). However, some of these silver-based treatments can become toxic to healthy cells and may cause negative side effects such as skin discoloration commonly known as agyria (Konop et al., 2016; Naik & Kowshik, 2017). Therefore, there is still a need for continued discovery of effective and affordable wound healing drugs and formulations. AgNPs were successfully synthesized using *Cotyledon orbiculata,* a popular medicinal plant in South Africa. The nanoparticles were shown to have good antimicrobial and anti-inflammatory properties (Tyavambiza, Elbagory, et al., 2021). Because the plant *C. orbiculata* has been used traditionally to treat wounds and skin infections, *C. orbiculata* synthesized nanoparticles may have enhanced wound healing activities. This study therefore focuses on the wound healing potential of *C. orbiculata* synthesized nanoparticles. It also investigates the cytotoxic effects of the nanoparticles at concentrations which promote wound healing.

Determining the cytotoxic effects of a wound healing agent or treatment is essential. Effective wound treatment should be toxic to bacteria only and not the surrounding healthy cells growing around the wound. Damage to fibroblast and keratinocytes (the most abundant cells at the wound site) impairs the wound healing process. It disrupts the epithelization process thus affecting wound closure. Some silver-based treatments such as silver sulfadiazine were reported to cause system toxicity resulting in the damage of organs such as the liver and kidneys (Cutting et al., 2007; Atiyeh et al., 2007; Adhya et al., 2015). It is therefore important to fully investigate the toxicity effects of any potential wound healing agent before declaring it as one. Hence in this study, the toxicity effects of *C. orbiculata* synthesized AgNPs to normal skin cells (KMST-6) were evaluated using mechanistic studies

such as apoptosis, TMRE (for mitochondrial depolarization), ROS (Reactive oxygen species) and toxicology gene expression studies.

1.2 Aims

- To determine the wound healing and cytotoxicity properties of *C. orbiculata* water extract and silver nanoparticles derived from this extract.

1.3 Main Objective

- To evaluate the wound healing properties of *C. orbiculata* water extract and its silver nanoparticles.
- To evaluate the cytotoxic effects of *C. orbiculata* water extract and its silver nanoparticles in skin fibroblasts, keratinocytes and epithelial cells.

1.4 Specific Objectives

- To prepare the *C. orbiculata* water extracts.
- To synthesize silver nanoparticles from the *C. orbiculata* water extract.
- To determine the phytochemicals, present in the *C. orbiculata* extracts and the silver nanoparticles.
- To evaluate the antioxidant properties of both the *C. orbiculata* water extracts and the silver nanoparticles.
- To evaluate the growth promoting and wound healing properties of the extracts and nanoparticles using KMST-6 (fibroblasts), HaCaT (keratinocytes) and CHO (epithelial cells) cell lines.
- To confirm wound healing effects of *C. orbiculata* water extracts and the silver nanoparticles by assessing the effects of the treatments on genes known to be involved in wound healing in the fibroblast cell line, KMST-6.
- To evaluate the cytotoxic effects of the extracts and nanoparticles on KMST-6, HaCaT and CHO cell lines using in-vitro assays which assess reactive oxygen species production, apoptosis, cellular uptake, and mitochondrial membrane potential.
- To evaluate the cytotoxic effects of the extracts and nanoparticles on KMST-6 cells by studying the effects of the treatments on genes known to be involved in toxicity.

1.5 Research Questions

- What are the main phytochemicals present in the different extracts of *C. orbiculata*?
- Are these phytochemicals also present in the *C. orbiculata* silver nanoparticles?
- What are the antioxidant and wound healing effects of *C. orbiculata* extracts and nanoparticles?
- How are the wound healing effects of the silver nanoparticles compared to that of the water extract?
- What are the molecular effects of the nanoparticles on wound healing in KMST-6 cells?
- Are the *C. orbiculata* plant extracts and nanoparticles toxic to KMST-6, HaCaT cells and CHO cells?
- If they are, which mechanism of toxicity do they use?
- Which toxicity genes are expressed (upregulated or downregulated) in the presence of *C. orbiculata* silver nanoparticles in KMST-6 cells?

1.6 Research rationale

Chronic wounds are a huge threat to global health systems. This is because of the increasing prevalence of risk factors associated with wounds, which include the development of antibiotic resistant microorganisms, diabetes and HIV. Even though there is a high prevalence (6.5 million) of people suffering from chronic wounds around the world, the number is constantly increasing. Chronic wounds are also associated with high mortality and morbidity rates. To worsen the situation, the care and treatment of chronic wounds is very difficult and expensive. It involves cleaning of the wound, applying different ointments, dressing the wound and maintaining a moist, bacteria free environment. This is difficult to attain as wounds can become infected with multidrug-resistant bacteria which are very difficult to eradicate. Advanced therapies based on growth factors, extracellular matrix and engineered skin can be used; however, they are very expensive and can cause undesirable side effects. Chronic wounds are a silent epidemic that causes discomfort and poses a huge financial burden to patients and health care systems.

Nanotechnology has been introduced in wound healing in an attempt to improve the healing of wounds. Nanomaterials, especially nanoparticles and nanocomposites, are being synthesized and incorporated in wound dressings and ointments. The common ones include

AgNPs and nanocomposites. This is due to the popularity of silver as a good antimicrobial agent. The most common commercial wound treatments containing silver include silver nitrate ($AgNO_{3)}$ and Silver sulfadiazine (SSD). These can however be toxic to healthy cells and can cause unwanted side effects. The synthesis of nanomaterials is therefore highly promising as they have been shown to be more effective and less toxic compared to SSD and $AgNO_3$. The synthesis of biogenic AgNPs further lessens the toxicity of nanoparticles. Biogenic synthesis of nanoparticles involves the use of biomaterials commonly plants, compared to the toxic chemicals which are used during chemical synthesis. Biogenic nanoparticles are more biocompatible and can therefore be easily and safely incorporated into biomedical applications compared to those that are chemically or physically synthesized.

C. orbiculata, a South African medicinal plant, has been used traditionally to treat acne, skin rashes and inflammation. It was proven scientifically to have antimicrobial, antioxidant and anti-inflammatory activities. AgNPs were also successfully synthesized using the water extract of this plant. The synthesized nanoparticles had enhanced antimicrobial and anti-inflammatory activity as compared to the water extract. Seeing the potential that the *C. orbiculata* synthesized nanoparticles and the water extract have, it is essential to investigate their potential wound healing activities as it might lead to the discovery of effective wound healing formulations. Therefore, this study investigated the wound healing, antioxidant and toxicity effects of *C. orbiculata* extract and nanoparticles.

References

Aboyewa, J.A., Sibuyi, N.R.S., Meyer, M. & Oguntibeju, O.O. 2021. Green Synthesis of Metallic Nanoparticles Using Some Selected Medicinal Plants from Southern Africa and Their Biological Applications. *Plants*, 10 (9): 1929. https://doi.org/10.3390/plants10091929

Adhya, A., Bain, J., Ray, O., Hazra, A., Adhikari, S., Dutta, G., Ray, S. & Majumdar, B.K. 2015. Healing of burn wounds by topical treatment : A randomized controlled comparison between silver sulfadiazine and nano - crystalline silver. *Journal of Basic and Clinical Pharmacy*, 6(1): 29–34. doi: 10.4103/0976-0105.145776

Atiyeh, B.S., Costagliola, M., Hayek, S.N. & Dibo, S.A. 2007. Effect of silver on burn wound

infection control and healing : Review of the literature. *Burns*, 33: 139–148. https://doi.org/10.1016/j.burns.2006.06.010

Cutting, K., White, R. & Edmonds, M. 2007. The safety and efficacy of dressings with silver – addressing clinical concerns. *International Wound Journal*, 4(2): 177-184. https://doi.org/10.1111/j.1742-481X.2007.00338.x

Dreifke, M.B., Jayasuriya, A.A. & Jayasuriya, A.C. 2015. Current wound healing procedures and potential care. *Materials Science and Engineering C*, 48: 651–662. https://doi.org/10.1016/j.msec.2014.12.068

Elbagory, A.M., Cupido, C.N., Meyer, M. & Hussein, A.A. 2016. Large scale screening of southern African plant extracts for the green synthesis of gold nanoparticles using microtitre-plate method. *Molecules*, 21: 1–20. https://doi.org/10.3390/molecules21111498

Frykberg, R.G. & Banks, J. 2015. Challenges in the Treatment of Chronic Wounds. *Advances in Wound Care*, 4(9): 560–582.

Guo, S. & Dipietro, L.A. 2010. Factors Affecting Wound Healing. (Mc 859): 219–229. DOI: 10.1177/0022034509359125

Iravani, S., Korbekandi, H., Mirmohammadi, S. V & Zolfaghari, B. 2014. Synthesis of silver nanoparticles : chemical , physical and biological methods. , 9 (December): 385–406.

Järbrink, K., Ni, G., Sönnergren, H., Schmidtchen, A., Pang, C., Bajpai, R. & Car, J. 2017. Prevalence and incidence of chronic wounds and related complications: A protocol for a systematic review. *Systematic Reviews*, 6(15): 1–7.

Jones, R.E., Foster, D.S. & Longaker, M.T. 2018. Management of Chronic Wounds — 2018. *Clinical Review and Education, JAMA*, 320(14): 1481–1482. doi:10.1001/jama.2018.12426

Khansa, I., Schoenbrunner, A.R., Kraft, C.T. & Janis, J.E. 2019. Silver in Wound Care—Friend or Foe?:A Comprehensive Review. *Plastic and Reconsructive Surgery Global Open*, 7(8): 1–10. doi: 10.1097/GOX.0000000000002390

Konop, M., Damps, T., Misicka, A. & Rudnicka, L. 2016. Certain Aspects of Silver and Silver Nanoparticles in Wound Care: A Minireview. *Journal of Nanomaterials*, 2016. 47. https://doi.org/10.1155/2016/7614753

Naik, K. & Kowshik, M. 2017. The silver lining : towards the responsible and limited usage of silver. *Journal of Applied Microbiology*, 123: 1068–1087. https://doi.org/10.1111/jam.13525

Rai, M.K., Deshmukh, S.D., Ingle, A.P. & Gade, A.K. 2012. Silver nanoparticles: The powerful nanoweapon against multidrug-resistant bacteria. *Journal of Applied Microbiology*,

112(5): 841–852. https://doi.org/10.1111/j.1365-2672.2012.05253.x

Siddique, S. & Chow, J.C.L. 2020. Application of Nanomaterials in Biomedical Imaging and Cancer Therapy. *Nanomaterials*, 10(1700): 1–40. https://doi.org/10.3390/nano10091700

Tyavambiza, C., Dube, P., Goboza, M., Meyer, S., Madiehe, A.M. & Meyer, M. 2021. Wound Healing Activities and Potential of Selected African Medicinal Plants and Their Synthesized Biogenic Nanoparticles. *Plants.* 10(12): 2635. https://doi.org/10.3390/plants10122635

Tyavambiza, C., Elbagory, A.M., Madiehe, A.M., Meyer, M. & Meyer, S. 2021. The Antimicrobial and Anti-Inflammatory Effects of Silver Nanoparticles Synthesised from Cotyledon orbiculata Aqueous Extract. *Nanomaterials*. 11(5): 1343. https://doi.org/10.3390/nano11051343

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to literature review

Medicinal plants are essential in the discovery of various medicinal compounds and drug formulations. They have been used traditionally to treat many human ailments including wounds, coughs and colds, digestive problems, diabetes mellitus, ulcers, cancers as well as hypertension and asthma (Oladeji, 2016; Maroyi, 2019). It was reported that about 25% of modern therapeutic drugs were derived from plants, this proves the reliability of plants as a source of drug discovery (Patel, 2014). Medicinal plants have been incorporated in the field of nanotechnology particularly in green nanotechnology. Nanotechnology is a field of science which deals with the synthesis, development and use of materials with sizes ranging in nanometres (Kavitha et al., 2013; Aboyewa et al., 2021). This technology can be applied in many fields such as biology and biomedicine, electronics, physics, material science and agriculture. In biomedicine, it has the potential to deliver improved methods for disease diagnostics and therapeutics. Green nanotechnology involves the synthesis of nanomaterials using bioactive agents such as plants, bacteria and fungi (Aboyewa et al., 2021). The synthesis of nanomaterials using medicinal plants produces bio-nanomaterials that are more biocompatible and thus more suitable for biomedical applications.

Many South African plants have been successfully used to synthesize different nanoparticles; among these plants is *Cotyledon orbiculata*. This is a shrubby succulent commonly known as pig's ears because of its oval shaped grey-green leaves. *Cotyledon orbiculata* is mostly distributed in the Western, Eastern and Northern Cape Provinces of South Africa (Mort et al., 2005). It is popular for its traditional uses in treating wounds, skin eruptions and abscesses, boils, burns, corns and warts, acne, earache and epilepsy (Maroyi, 2019). *Cotyledon orbiculata* is known to have antimicrobial, anti-inflammatory, antioxidant, anticonvulsant and antinociceptive activities (Kumari et al., 2016; Tyavambiza et al., 2021; Amabeoku & Kabatende, 2012; Abubakar et al., 2013).

C. orbiculata was successfully used to synthesize silver nanoparticles. The synthesized nanoparticles were shown to have enhanced antimicrobial and anti-inflammatory effects compared to the *C. orbiculata* water extract (Tyavambiza et al., 2021). The traditional uses of *C. orbiculata* suggest that it has wound healing properties. However, the wound healing properties of this plant and its nanoparticles are yet to be explored. Whereas the wound healing properties of other African plants and their nanoparticles have been reported (Chai et al., 2018; Singla, Soni, Patial, et al., 2017; Paul & Londe, 2018; Singla, Soni, Markand, et

al., 2017). This chapter will focus on the properties of a good wound healing agent and the wound healing potential of plants and their synthesized biogenic materials. It is divided into 2 review articles with the titles, "*Wound Healing Activities and Potential of Selected African Medicinal Plants and Their Synthesized Biogenic Nanoparticles*" and "*Cellular and molecular events of wound healing and the potential of silver based nanoformulations as wound healing agents*" respectively.

2.1.1 References

Aboyewa, J.A., Sibuyi, N.R.S., Meyer, M. & Oguntibeju, O.O. 2021. Green Synthesis of Metallic Nanoparticles Using Some Selected Medicinal Plants from Southern Africa and Their Biological Applications. *Plants*, 10(9): 1929. https://doi.org/10.3390/plants10091929

Abubakar, K., Abdulkadir, R., Ear, N. & Centre, C. 2013. Evaluation of the Anticonvulsant Effect of the Methanol Extract of Evolvulus alsinoides in Mice. *Scholars Academic Journal of Pharmacy*. 2(6): 436–441.

Amabeoku, G.J. & Kabatende, J. 2012. Antinociceptive and anti-inflammatory activities of leaf methanol extract of cotyledon orbiculata L. (Crassulaceae). *Advances in Pharmacological Sciences*. 2012: 1-6. https://doi.org/10.1155/2012/862625

Chai, S., Wang, Y., Qiao, Y., Wang, P., Li, Q., Xia, C. & Ju, M. 2018. Bio fabrication of silver nanoparticles as an effective wound healing agent in the wound care after anorectal surgery. *Journal of Photochemistry & Photobiology, B : Biology*. 178(67): 457–462. https://doi.org/10.1016/j.jphotobiol.2017.10.024

Kavitha, K., Baker, S., Rakshith, D., Kavitha, H., Yashwantha Rao, H., Harini, B. & Satish S. 2013. Plants as Green Source towards Synthesis of Nanoparticles. *International Research Journal of Biological Sciences*, 2(6): 66–76.

Kumari, A., Baskaran, P. & Van Staden, J. 2016. In vitro propagation and antibacterial activity in Cotyledon orbiculata: a valuable medicinal plant. *Plant Cell, Tissue and Organ Culture*, 124(1): 97–104. DOI 10.1007/s11240-015-0878-0

Maroyi, A. 2019. A review of botany, medicinal uses, phytochemistry and biological activities of Cotyledon orbiculata. *Journal of Pharmaceutical Sciences and Research*, 11(10): 3491–3496.

Mort, M.E., Levsen, N., Randle, C.P., Jaarsveld, E. Van, Palmer, A., Archibald, J., Burgoyne, P. & Crawford, D. 2005. Phylogenetics and diversification of cotyledon (crassulaceae)

inferred from nuclear and chloroplast dna sequence DATA Botanical Garden staff for providing plant materials and Jenny Archibald for field assistance. *American Journal of Botany*, 92(7): 1170–1176.

Oladeji, O. 2016. The Characteristics and Roles of Medicinal Plants : Some Important Medicinal Plants in Nigeria. *Natural Products : An Indian Journal*, 12(3): 1–8.

Patel, D. 2014. Some Traditional Medicinal Plants Useful for Boil, Burn and for Wounds Healing. *Journal of Biodiversity & Endangered Species*. 2(4): 2–5. doi:10.4172/2332-2543.1000133

Paul, M. & Londe, Y.V. 2018. Pongamia pinnata seed extract - mediated green synthesis of silver nanoparticles : Preparation , formulation and evaluation of bactericidal and wound healing potential. *Applied Organometallic Chemistry*. 33(3): 1–9. https://doi.org/10.1002/aoc.4624

Singla, R., Soni, S., Markand, P., Kumari, A., Mahesh, S., Patial, V., Padwad, Y.S. & Yadav, S.K. 2017. In situ functionalized nanobiocomposites dressings of bamboo cellulose nanocrystals and silver nanoparticles for accelerated wound healing. *Carbohydrate Polymers*. 155(2017): 152–162. http://dx.doi.org/10.1016/j.carbpol.2016.08.065

Singla, R., Soni, S., Patial, V., Markand, P., Kumari, A., Mahesh, S., Padwad, Y.S. & Yadav, S. 2017. International Journal of Biological Macromolecules In vivo diabetic wound healing potential of nanobiocomposites containing bamboo cellulose nanocrystals impregnated with silver nanoparticles. *International Journal of Biological Macromolecules*, 105(2017): 45–55. http://dx.doi.org/10.1016/j.ijbiomac.2017.06.109

Tyavambiza, C., Elbagory, A.M., Madiehe, A.M., Meyer, M. & Meyer, S. 2021. The Antimicrobial and Anti-Inflammatory Effects of Silver Nanoparticles Synthesised from Cotyledon orbiculata Aqueous Extract. *Nanomaterials*. 11(5): 1343. https://doi.org/10.3390/nano11051343

CHAPTER 2.1

PUBLISHED REVIEW MANUSCRIPT: Wound Healing Activities and Potential of Selected African Medicinal Plants and Their Synthesized Biogenic Nanoparticles

plants

MDPI

Review

Wound Healing Activities and Potential of Selected African Medicinal Plants and Their Synthesized Biogenic Nanoparticles

Caroline Tyavambiza [1,2], Phumuzile Dube [2], Mediline Goboza [2], Samantha Meyer [1], Abram Madimabe Madiehe [2,3] and Mervin Meyer [2,*]

1 Phytotherapy Research Group, Department of Biomedical Sciences, Cape Peninsula University of Technology, P.O. Box 1906, Bellville, Cape Town 7535, South Africa; carolinetyavambiza@gmail.com (C.T.); meyers@cput.ac.za (S.M.)
2 DSI/Mintek Nanotechnology Innovation Centre (NIC), Biolabels Node, Department of Biotechnology, University of the Western Cape, Private Bag X17, Bellville, Cape Town 7535, South Africa; phumuedube@gmail.com (P.D.); 3990471@myuwc.ac.za (M.G.); amadiehe@uwc.ac.za (A.M.M.)
3 Nanobiotechnology Research Group, Department of Biotechnology, University of the Western Cape, Private Bag X17, Bellville, Cape Town 7535, South Africa
* Correspondence: memeyer@uwc.ac.za; Tel.: +27-21-9592032

Abstract: In Africa, medicinal plants have been traditionally used as a source of medicine for centuries. To date, African medicinal plants continue to play a significant role in the treatment of wounds. Chronic wounds are associated with severe healthcare and socio-economic burdens despite the use of conventional therapies. Emergence of novel wound healing strategies using medicinal plants in conjunction with nanotechnology has the potential to develop efficacious wound healing therapeutics with enhanced wound repair mechanisms. This review identified African medicinal plants and biogenic nanoparticles used to promote wound healing through various mechanisms including improved wound contraction and epithelialization as well as antibacterial, antioxidant and anti-inflammatory activities. To achieve this, electronic databases such as PubMed, Scifinder® and Google Scholar were used to search for medicinal plants used by the African populace that were scientifically evaluated for their wound healing activities in both in vitro and in vivo models from 2004 to 2021. Additionally, data on the wound healing mechanisms of biogenic nanoparticles synthesized using African medicinal plants is included herein. The continued scientific evaluation of wound healing African medicinal plants and the development of novel nanomaterials using these plants is imperative in a bid to alleviate the detrimental effects of chronic wounds.

Keywords: African medicinal plants; biogenic nanoparticles; wound healing; antibacterial; antioxidant; anti-inflammatory

Citation: Tyavambiza, C.; Dube, P.; Goboza, M.; Meyer, S.; Madiehe, A.M.; Meyer, M. Wound Healing Activities and Potential of Selected African Medicinal Plants and Their Synthesized Biogenic Nanoparticles. *Plants* **2021**, *10*, 2635. https://doi.org/10.3390/plants10122635

Academic Editors: Ki Hyun Kim and Franklin Gregory

Received: 30 September 2021
Accepted: 10 November 2021
Published: 30 November 2021

1. Introduction

1.1. Wounds

Wounds are defined as injury to living tissue which leads to the disruption of its normal anatomical structure and function [1]. They arise due to physical, chemical, thermal, microbial or immunological damage to the tissue [2]. Regardless of the aetiology and type, wounds can cause damage to the tissue and disrupt the surrounding environment. The damage can affect the integrity of the skin epithelial layer and can also extend into the subcutaneous tissue disrupting other structures such as tendons, muscles and nerves [1,3]. Failure of wounds to heal normally leads to chronic wounds. Chronic wounds are a silent epidemic claiming the lives of many individuals worldwide. In the past decade, it has been estimated that 6 million people suffer from chronic wounds worldwide [2,4–6]. Wounds have a significant negative impact on the economic and social lives of patients and their families. They cause severe pain, physical disability such as immobility and loss of function, loss of self-esteem, depression and anxiety as well as premature death [7,8].

Not only do wounds affect the patients' social life, they also pose a great financial burden on patients and the healthcare systems. Chronic wounds consume a great amount of healthcare resources around the world [8].

Chronic wounds are predominantly a condition of the elderly, however, their prevalence is expected to increase in all age groups because of the increase in conditions that impede wound healing, such as diabetes, obesity and vascular disorders [9,10]. The high and increasing prevalence of these conditions continues to increase the global burden of chronic wounds. Unfortunately, to our knowledge, there are no statistical records on the prevalence of chronic wounds in Africa.

1.2. Classification of Wounds

Wounds can be classified depending on their healing time. Acute wounds heal timely, following the normal wound healing process. They result in the restoration of anatomical and functional integrity of the normal tissue [11]. On the other hand, chronic wounds require prolonged time to heal, and they do not progress through the normal stages of wound healing [12]. Given their complexity, chronic wounds tend to fall into four categories, namely pressure ulcers, venous ulcers, diabetic ulcers and arterial insufficiency ulcers [8,13].

Wounds can also be classified as open (those associated with disruption and discontinuity of the skin) or closed (when there is damage to the underlying tissue but the skin is intact) [12]. Open wounds can be further classified as incisions (cut by a sharp object such as a scalpel), lacerations (tear like wounds), abrasions (scraping of the outer skin layer), puncture wounds, penetration wounds (caused by an object entering or exiting the skin surface) and gunshot wounds. Most open wounds are often associated with a wide range of bacterial and fungal infections as the underlying tissues are exposed to the outside environment [5,12]. The challenge of antimicrobial resistance in treating these infections increases the complications and burden of these wounds [14,15]. Repetitive trauma, which is common in diabetic foot ulcers, can also delay or even stop the wound healing process [16]. Closed wounds include contusions (caused by a blunt force trauma that damages tissue under the skin), haematomas (accumulation of blood under skin due to a damaged blood vessel) and crush injuries (occurs when great external force is applied on the skin over a long period of time) [17].

1.3. Phases of Wound Healing

Wound healing is a complex physiological process which aims to restore the anatomical structure and function of an injured tissue [18]). This process is divided into four integrated phases, namely haemostasis, inflammation, proliferation and tissue remodelling. Haemostasis involves vascular constriction and coagulation. Platelets attach to exposed collagen surfaces and extracellular matrix leading to the activation of the coagulation cascade and clot formation, thereby preventing blood loss [19,20]. This process is mediated by growth factors released by platelets such as Epidermal growth factor (EGF), Platelet-derived growth factor (PDGF), Transforming growth factor-beta (TGF-β) and Fibroblast growth factor (FGF). These growth factors together with some cytokines play a vital role in the movement of monocytes and neutrophils to the wound site initiating the inflammatory phase [5,21]. During inflammation, neutrophils and monocytes penetrate the wound site. Neutrophils, which are the first leukocytes to infiltrate the wound site, eliminate invading microorganisms through phagocytosis and release reactive oxygen species and proteolytic enzymes [22,23]. Neutrophils also release IL-8 aiding chemotaxis which attracts monocytes and other cells to the wound site [19]. Monocytes then differentiate into macrophages which continue the process of phagocytosis and cleansing of the wound. They also initiate the development of granulation tissue and angiogenesis by releasing various growth (FGF, EGF, TGF-b, and PDGF) and cytokines (IL-1 and IL-6) [24,25].

The proliferation phase comes immediately after the cleansing of the wound and then the repair process (building of new tissue). This phase is characterized by the formation

of granulation tissue, angiogenesis, wound contraction and epithelialization [26]. It involves the activity of many cells including fibroblasts, keratinocytes, and endothelial cells. Granulation involves the deposition of newly synthesized extracellular matrix composed of collagen, fibrin and fibronectin which replaces the damaged tissue [27]. Angiogenesis by endothelial cells ensures sufficient supply of nutrients and oxygen to the granulation tissue [28]. Fibroblasts and myofibroblasts cause the wound to contract by pulling the wound edges together. Subsequently keratinocytes migrate over the granulation tissue from the wound edges towards the center to cover the wound and protect the underlying issue [21,29]. The tissue remodeling phase (last phase) occurs after the wound has closed. This phase aims to achieve maximum tensile strength and flexibility of the new tissue. However, the wound never achieves the same level of tensile strength as the normal tissue. Remodeling and reorganization of collagen fibres occurs, resulting in the formation of cross-linkages which reduces scar thickness. The level of vascularity decreases as the scar matures [30,31]. The cells playing a part in wound repair are removed by apoptosis as they are no longer needed [32].

1.4. Risk Factors and Conditions Associated with Wound Healing

The wound healing process is not only complex but also fragile, as it is susceptible to numerous interruptions and failure. Various factors can affect one or more phases of wound healing as well as its sequence and time frame, leading to the impairment of this process. These factors delay wound healing resulting in poor outcomes thereby increasing the patient morbidity and mortality [26]. The risk factors of wound healing are classified into local and systemic. Systemic factors affect the general health of an individual, which influences their ability to heal normally. These factors include age, stress, chronic diseases (such as diabetes mellitus, hepatic and renal failure), obesity, alcoholism, medication and immunocompromising conditions (such as cancer and AIDS) [15]. Local factors are those that directly influence the characteristics of the wound itself. These include infection, oxygenation, venous insufficiency and the presence of foreign material in the wound. All these factors are related, usually systemic factors act through the local factors in affecting wound healing [33].

Infections are one of the most common complications of wounds, as they disrupt the normal wound healing process, leading to the development of chronic wounds. Frequently isolated bacterial species in wounds include *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *β-hemolytic streptococci* [15,34]. The loss of skin integrity as a result of the injury enables pathogens to invade the wound site and cause infections. Bacterial invasion affects all stages of wound healing, particularly the inflammatory phase. This phase plays an important role in microbial clearance and wound cleansing. However, in the presence of an infection, inflammation is prolonged due to the increased microbial load and incomplete microbial clearance [35]. Bacterial toxins decrease chemotaxis and increase the secretion of pro-inflammatory cytokines such as IL-1 and TNF-α. This further prolongs the inflammation, causing the wound to enter a chronic state [25,34]). The continuous inflammatory phase increases the presence of neutrophils, which will lead to the degradation of the extracellular matrix and loss of important wound healing growth factors (TGF-β and PDGF) [36]. The increase in neutrophils will also increase the production of reaction oxygen species (ROS). The ROS are essential in cellular metabolism, however, when produced in excess they cause cellular damage and further affect the healing of the wound [11]. In the treatment of wounds, it is therefore imperative for a wound healing agent to have antimicrobial, anti-inflammatory and antioxidant activities as this will aid in the healing of the wound.

1.5. Conventional Treatment and Management of Wounds

The care and management of wounds is of great importance in wound healing, as it ensures and aids proper wound healing. The aim of good wound management is to provide and maintain a warm, moist, non-toxic environment which supports natural wound healing.

Poorly managed wounds can lead to the development of non-healing chronic wounds [37]. Hygiene is one of the important factors in wound management as it minimizes the risk of wound infections. Wounds undergo debridement before the application of topical treatments such as antimicrobial and silver wound dressings, silver sulfadiazine creams and oils and sprays. Debridement is the removal of necrotic tissue from the affected area in-order to promote wound healing [20]. Topical therapies are the first line of treatment of wounds. After assessment, and if healing is not achieved, advanced wound therapies are then used. The most common advanced wound therapies include negative pressure wound therapy, administration of growth factors, hyperbaric oxygen and skin grafts [8].

These therapies are very costly, hence, not all individuals can access them, especially in poor resource countries. They are also associated with severe complications such as bleeding, infection, barotrauma and oncogenesis [38–40]. Although these therapies are available, the burden of chronic wounds is increasing. The search for new affordable and easily accessible wound treatment drugs and therapies is therefore vital. One reliable source of drug discovery is medicinal plants as they have been used traditionally for many years in the treatment of many human ailments. It has been reported that 25% of modern drugs were derived from plants [41,42]. Nanomaterials synthesized from medicinal plants were also shown to be beneficial in wound healing. In this article, we will review different wound healing mechanisms of African medicinal plants as well as those of biogenic nanoparticles synthesized using African medicinal plants. To achieve this, electronic databases such as PubMed, Scifinder® and Google Scholar were used in the search. The African medicinal plants and their biogenic nanoparticles were evaluated for their wound healing activities in both in vitro and in vivo models from 2004 to 2021.

2. African Medicinal Plants in the Treatment of Wounds

The African continent is well known for an immense biodiversity distribution with nearly 45,000 different plant species of which about 5000 species have been used as traditional medicines [5]. Throughout history, medicinal plants have been used exclusively in the treatment and management of different diseases, especially in poor communities. It is documented that 80% of the African populace rely on medicinal plants as their primary source of medication as medicinal plants are more affordable, easily accessible and are associated with fewer side effects when compared to conventional medicines [43]. Medicinal plants are a good source of compounds which could serve as leads for drug discovery for wound healing [44]. The wound healing activities displayed by medicinal plants are attributed to the presence of bioactive chemicals such as phenols, alkaloids, triterpenes, and flavonoids. In wound healing, these bioactive compounds have been reported to have antioxidant and antimicrobial activities, improve collagen deposition and increase the proliferation of both fibroblasts and keratinocytes [5,45]. In most instances, the wound healing process progresses naturally. However, the presence of an infection can cripple the healing process by lengthening the inflammatory phase, disturbing the normal clotting mechanisms, disrupting leukocyte function and angiogenesis [46]. Thus, the antimicrobial effects exhibited by many plants explain the significant roles they display in the healing of wounds. On the other hand, phenolic compounds were reported to play an important role in the healing of wounds because of their antioxidant effect against free radicals which negatively influence the progression of wound healing [47]. Various reports have provided information on plants used in Africa for the treatment of wounds. Some of these plants are listed in Table 1 below.

Table 1. Wound healing activities of selected African medicinal plants.

Plant Name	Family	Plant Part Used	Mode of Wound Healing	Reference
Acacia senegal	*Mimosae*	Root	Antimicrobial	[48]
Aloe ferox	*Asphodelaceae*	Leaf gel	Antioxidant, anti-inflammatory and antibacterial activities	[49,50]
Aloe vera	*Asphodelaceae*	Leaf gel	Enhance mature granulation, antioxidant, anti-inflammatory and antibacterial activities	[45]
Aspalathus linearis	*Fabaceae*	Leaves	Antioxidant, anti-inflammatory and antibacterial activities	[51]
Argyreia nervosa	*Convolvulaceae*	Leaves and roots	Antibacterial and anti-inflammatory	[52,53]
Boerhavia diffusa	*Nyctaginaceae*	Whole plants	Increased myoblast migration, antioxidant	[44]
Boophone disticha	*Amaryllidaceae*	Bulb	Antimicrobial, anti-inflammatory	[53,54]
Bridelia ferruginea	*Combretaceae*	Leaf	Stimulation of fibroblasts	[55]
Bulbine frutescens	*Asphodelaceae*	Gel sap	Improvement of wound contraction	[56]
Catharanthus roseus	*Apocynaceae*	Leaf	Improved wound contraction, decreased epithelization period and antibacterial activities	[57]
Centella asiatica	*Umbelliferae*	Leaves	Increased cellular proliferation, angiogensis collagen synthesis, antioxidant and anti-inflammatory	[58,59]
Cotyledon orbiculata	*Crassulaceae*	Leaf gel	Antioxidant, anti-inflammatory and antibacterial activities	[60–63]
Ficus asperifolia	*Moraceae*	Bark	Stimulation of fibroblasts, antioxidant	[46]
Gossypium arboreum	*Malvaceae*	Leaf	Stimulation of fibroblasts, antioxidant	[46]
Gunnera perpensa	*Gunneracea*	Root	Antioxidant and antibacterial activities	[64,65]
Gymnosporia senegalensis	*Celestraceae*	Leaf, bark, roots	Anti-inflammatory and antimicrobial activities	[66]
Haemanthus coccineus	*Amaryllidaceae*	Bulb	Anti-inflammatory	[58,67]
Maytenus heterophylla	*Celastraceae*	Crushed leaves	Anti-inflammatory and antimicrobial activities	[68]
Merwilla natalensis	*Asparagaceae*	Fresh and burnt bulb scales	Anti-inflammatory and antimicrobial activities	[50]
Ocimum gratissimum L.	*Lamiaceae*	Leaf	Antibacterial and activities	[55,69]
Parkia biglobosa	*Fabaceae*	Stem	Stimulation of fibroblasts and antibacterial activity	[55]
Sutherlandia frutescens	*Fabaceae*	Leaves	Antibacterial	[70]
Tecoma capensis	*Bignoniaceae*	Bark	Improved wound contraction and re-epithelialization	[71]
Terminalia sericea	*Combretaceae*	Bark	Antioxidant, anti-inflammatory and antibacterial activities	[64,72]
Vernonia amygdalina	*Asteraceae*	Leaf	Improved wound contraction and re-epithelialization	[73]

3. Nanotechnology as a Wound Healing Intervention

Medicinal plants have been incorporated in the field of nanotechnology in a bid to enhance the activity of the plants. Different nanoparticles including silver [63,70,74,75], gold [76,77], titanium dioxide [78] and selenium [79,80] have been successfully synthesized using plants.

Silver containing wound therapies have a long history of use in the treatment of chronic wounds. Silver releasing agents are known to inhibit the manifestation of a variety of microorganisms including fungi, Gram-positive and Gram-negative bacteria on wounds [81]. This is due to silver interfering with the electron transport system or binding to and inhibiting DNA replication of the microbes [82]. Additionally, these silver therapeutics display low systemic toxicity as they can be neutralized by anions in body fluids. Some of the most widely used silver based wound dressings, such as Askina Calgitrol Ag®, Aquacel® Ag Dressing, 3M Tegaderm Alginate Ag Silver Dressing and Systagenix Silvercel antimicrobial alginate dressing, contain silver alginates, whilst common topical agents such as Silvadene, Thermazene® and SSD Cream® contain silver sulfadiazine [83–88]. Certain dressings like Aquacel® Ag Dressing may cause skin irritation and allergies [86]. The more extensive methods of chronic wound treatment such as negative pressure wound therapy, administration of growth factors, hyperbaric oxygen and skin grafts have been reported to be costly, unsafe and detrimental to surrounding healthy tissues [39]. Hence, novel wound

healing strategies with increased efficacy and limited side effects are constantly being developed. In this line, nanotechnology has the potential to address these matters of concern, as it is providing novel materials that exhibit enhanced size-dependent properties [27,89,90].

Nanotechnology has played a notable role in the delivery of improved methods for disease diagnostics and therapeutics. To date, about 51 nanopharmaceuticals synthesized from liposomes, polymers, nanocrystals, proteins, micelles and inorganic reducing agents have been approved by the US Federal Drug Agency and are available in clinical practices, whilst several nanomedicines are undergoing clinical trials [91,92]. Examples include the widely used Acticoat^{TM} Flex 3 and Acticoat©, which are silver nanoparticle-based products that promote wound healing whilst reducing pain and infection at the wound site [93,94]. Silver nanoparticles have been shown to promote wound healing with improved cosmetic appearance. The activity has been attributed to their antimicrobial properties, reduction in wound inflammation and modulation of fibro-genic cytokines. Tian et al. (2007) reported that silver nanoparticles had an increased healing rate compared to silver sulfadiazine, the standard treatment for burn wounds [95]. Despite their popularity, the prolonged use of these silver based wound therapies may slow the wound healing process due to epithelial and fibroblast cytotoxicity and cause negative cosmetic effects such as argyria. Other nanoparticles such as gold, zinc oxide and selenium have also been used to promote wound healing [96,97]. Thermo-responsive gels containing gold nanoparticles displayed antimicrobial and wound healing properties as shown by in vitro, in vivo and histopathological assays [98]. Low concentrations of gold nanoparticles, 34 nm in size, significantly promoted the proliferation of keratinocytes, which are important in wound closure [99]. This proved the great efficiency and potential of the gold nanoparticles as wound therapeutics and a probable alternative to autografts.

3.1. Biogenic Nanoparticles in Wound Healing

Although several chemical and physical synthesis methods for the production of nanoparticles are known and used, the synthesis of nanoparticles using biological models has several advantages over these methods. This is due to the fact that nanoparticle synthesis using physio-chemical approaches utilizes organic solvents and toxic reducing agents whilst producing hazardous waste-products [100]. The biologically synthesized nanoparticles possess a great advantage where applicability in industrial and human health care products is concerned. Numerous biological approaches which employ different natural resources such as plants, microorganisms and algae in the synthesis of nanoparticles are available [77]. Although microorganisms are widely used to reduce metallic salt solutions to form corresponding nanoparticles, the process uses toxic and costly reactants that result in an unsuitable platform for biocompatible synthesis [27]. On the other hand, plants are readily used to synthesize nanoparticles as they are easily accessible and can promote rapid nanoparticle synthesis [101]. Bioactive plant phytochemicals (alkaloids, phenolic acids, polyphenols, saponins, proteins, sugars, and terpenoids) are believed to have significant roles in the synthesis of nanoparticles [102]. They are involved in the reduction of metallic ions and also act as capping agents that stabilize the synthesized nanoparticles [103].

The unique features of synthesized nanoparticles such as large surface area to volume ratio allow them to bind and deliver small-sized beneficial compounds to target sites. The ability of biogenic nanoparticles to combine with bioactive drugs and other useful compounds can result in a synergistic effect, which further enhances their efficiency and effectiveness. Combining *Urtica dioica* silver nanoparticles and the antibiotics Amoxicillin, Amikacin, Kanamycin, Tetracycline and Cefotaxime showed a synergistic inhibitory activity against *S. aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Bacillus subtilis*, *Serratia marcescens* and *Salmonella typhimurium.* This was shown by a 0.1- to 17.8-fold increase in the zones of inhibition [104]. Many polymers including chitosan, hyaluronan, pyridoxine and collagen have been combined with silver nanoparticles to form silver nanocomposites with enhanced wound healing properties [97]. The combi-

nation of silver nanoparticles with tetracycline was shown to significantly improve wound healing and wound infection control in Albino mice [105].

Given the history and benefits of medicinal plants found on the African continent, combining these plants and nanotechnology can lead to the development of important nanomedicines. Though some of the medicinal plants are not native to the African continent, several are found and harvested in one or more African countries. The bio-synthesized nanoparticles may potentially improve wound healing efficacy, exhibit better antimicrobial activity and/or promote accelerated wound closure compared to the raw materials and current treatments as shown in Table 2. The enhanced activity could be attributed to the expected increase in the stability of bioactive phytochemicals within the nanoparticles and the large surface area of the synthesized nanoparticles [75,106,107].

Table 2. Biogenic nanoparticles synthesized using African medicinal plants exhibiting wound healing activities.

Plant Name	Nanoparticle Type	Particle Size	Experimental Outcomes	Reference
Azadirachta indica	Silver	60–85 nm	(a) In diabetic rats, the nanoparticles show increased wound contraction and accelerated wound healing compared to the control and the drug alone.	[108]
		33 nm	(b) Synthesized silver nanoparticles displayed enhanced antibacterial and antioxidant effects when compared to the leaf extracts alone. The nanoparticle infused PF127 hydrogel improved the wound contraction rate in mice.	[109]
		30 nm	(c) The wound beds where the nanoparticles were topically applied showed no microbial growth, haemorrhage, or formation of pus throughout treatment. The nanoparticle treated female BALB/c mice showed better wound-healing capacity when compared to control group animals.	[74]
Cassia auriculata L.	Silver	15–90 nm	Nanoparticles showed an increased percentage of wound contraction in both excision and incision wound models when compared to the drug Povidone iodine as well as the *C. auriculata* extracts.	[110]
Catharunthus roseus	Silver	20 nm	(a) When the silver nanoparticles were topically applied on open wounds, the treated animals exhibited better wound healing activity and decreased inflammation compared to those treated with the respective aqueous plant extract.	[111]
		30 nm	(b) Silver nanoparticle treated wounds on female BALB/c mice showed decreased irritation with greater wound healing capacity when compared to the positive control.	[74]
Curcuma longa L.	Silver	15–40 nm	Synthesized nanoparticles increased fibroblast cell proliferation and migration indicating effective wound healing.	[112]
Falcaria vulgaris	Silver	40–45 nm	The nanoparticles promoted the healing of cutaneous wounds on male rats by decreasing the wound area and increasing wound contracture, antimicrobial and antioxidant activity, fibroblast-fibrocyte ratio and macrophages at the wound site when compared to the activity of *F. vulgaris* extracts and tetracycline.	[113]
Mimosa pudica	silver	7.63 nm	Biosynthesized nanoparticles incorporated in an electrospun polyvinyl alcohol (PVA) improved keratinocyte migration when compared to the activity of PVA alone.	[114]
Moringa oliefera	Titanium dioxide	100 nm	The synthesized nanoparticles promoted significant wound closure in Albino rats when compared to a standard commercial wound therapeutic.	[75]
Prosopis juliflora	Silver	10–20 nm	The percentage of wound healing was increased for the nanoparticles when compared to that of Povidine-iodine.	[115]
Woodfordia fruticosa	Gold	13 nm	Topical application of the synthesized nanoparticles on excised wounds on Wistar albino rat models resulted in the effective prevention of microbial adhesion and accelerated wound closure in comparison to the activity of 5% Povidone-iodine.	[116]

3.2. Biological Nanoparticles with Potential Wound Healing Properties

Delonix elata copper oxide nanoparticles (36 nm in size) applied on human wounds after anal rectum surgery caused rapid wound epithelialization [117]. Nanoparticle treated wounds also showed a weaker inflammatory response probably resulting from cytokine modulation. An association between wound healing and the anti-inflammatory and/or antioxidant activities of therapeutics has been established. Anti-inflammatory agents are important in controlling inflammation whilst antioxidants address oxidative stress at the site of the wound thereby accelerating wound healing [118]. Both these properties have also been reported to decrease the production of pus at the wound site. Numerous nanoparticles synthesized using medicinal plants found on the African continent have been shown to exhibit either antioxidant and/or anti-inflammatory activity (Table 3), classifying them as potential wound healing therapies.

Table 3. Biogenic nanoparticles synthesized using African medicinal plants with bioactivities that promote wound healing.

Plant Name	Nanoparticle	Size	Anti-Inflammatory Activity	Antioxidant Activity	Reference
Allium ampeloprasum	Silver	8 nm	-	Yes	[119]
Aloe vera	Selenium	7–48 nm	-	Yes	[80]
Areca catechu	Silver	18.2–24.3 nm	-	Yes	[120]
Artocarpus altilis	Silver	38 nm	-	Yes	[121]
Cola nitida	Silver	12–80 nm	-	Yes	[122]
Cotyledon orbiculata	Silver	20–60 nm	Yes	-	[63]
Cuminum cyminum	Silver	-	Yes	-	[123]
Garcinia indica	Gold	20–30 nm	-	Yes	[124]
Garcinia indica	Silver	5–30 nm	-	Yes	[125]
Helicteres isora	Silver	38.23 nm	-	Yes	[126]
Hypoxis hemerocallidea	Gold	27 nm	Yes	-	[127]
Kalanchoe pinata	Zinc oxide	24 nm	Yes	-	[128]
Nigella sativa	Silver	25.2 nm	Yes	Yes	[129]
Oxalis corniculata	Silver	30 nm	-	Yes	[130]
Phoenix dactylifera	Zinc sulphide	<70 nm	-	Yes	[131]
Terminalia bellirica	Silver	11 nm	Yes	Yes	[132]
Terminalia bentazoe	Silver	7 nm	Yes	Yes	[132]
Terminalia catappa	Silver	10 nm	Yes	Yes	[132]
Terminalia mellueri	Silver	10 nm	Yes	Yes	[132]
Zingiber officinale	Selenium	100–150 nm	-	Yes	[79]

The potential of biogenic nanoparticles, especially those synthesized using African medicinal plants has not been fully explored. In many instances biogenic nanomaterials have been proven effective for most biomedical applications. Numerous nano-based medicines have been approved by the FDA for use while many others are on trial. It is therefore of great importance to investigate all wound healing activities of biogenic nanoparticles as this might lead to the discovery of efficacious wound healing therapeutics.

4. Conclusions and Recommendations

A thorough and effective wound healing process is crucial to the well-being and socio-economic state of patients. The complexity of a typical healing process is due to the various components involved at both cellular and subcellular levels. Although clas-

sic topical wound therapeutics including medicinal plants and silver sulfadiazine are still common among the African populace, the prevalence of chronic wounds remains high. Henceforth, current technologies such as nanotechnology should focus mainly on the molecular and cellular aspects of wound healing in the development of therapeutic interventions. Considering that silver nanoparticle impregnated wound dressings are slowly becoming a standard in wound and burn therapy, the discovery of the full potential of nanomedicine is necessary. Unlike conventional physio-chemical methods, the green synthesis of nanoparticles using known medicinal plants is a non-toxic approach in the production of biocompatible nanoparticles. It is also cost effective, making it affordable for low resource African countries to utilize these strategies to develop alternative treatments. Given the great potential of biogenic nanoparticles in various biomedical applications and medicinal plants in drug discovery, it is imperative that widely used wound healing medicinal plants native to the African continent be incorporated in nanomaterials. However, the scarcity of scientific evidence reporting the wound healing activities of biogenic nanoparticles synthesized using these African medicinal plants is concerning. Green nanotechnology still has a vast selection of raw materials still to be explored on the African continent. Therefore, the development of novel nanomaterials exhibiting impressive wound healing activity and the further understanding of the mechanisms underlying their beneficial properties is important for future research.

Author Contributions: Conceptualization, M.M.; writing—original draft preparation, C.T., P.D. and M.G.; writing—review and editing, C.T., P.D., M.G., S.M., A.M.M. and M.M.; supervision, S.M., A.M.M. and M.M.; project administration, C.T., P.D. and M.G.; funding acquisition, S.M. and M.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the South African National Research Foundation (grant number 114598 and 106642) and the DSI/MINTEK Nanotechnology Innovation Centre (NIC) and "The APC was funded by DSI/MINTEK Nanotechnology Innovation Centre (NIC)".

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Velnar, T.; Bailey, T.; Smrkolj, V. The Wound Healing Process: An Overview of the Cellular and Molecular Mechanisms. *J. Int. Med. Res.* **2009**, *37*, 1528–1542. [CrossRef] [PubMed]
2. Abubacker, M.N.; Devi, P.K. Evaluation of Wound Healing Activity of Ethanolic Extract of *Lepidagathis Cristata*. Willd. (Acanthaceae) in Experimental Animal models. *Eur. J. Pharm. Med. Res.* **2016**, *3*, 410–416.
3. Kayode, A.O. Epidemiological Study on Wound Distribution Pattern in Horses Presented at Two Veterinary Clinics in South West, Nigeria between 2007–2010. *J. Dairy Vet. Anim. Res.* **2017**, *5*, 127–129.
4. Barku, V. Wound Healing: Contributions from Medicinal Plants and Their Phytoconstituents. *Annu. Res. Rev. Biol.* **2018**, *26*, 1–14. [CrossRef]
5. Agyare, C.; Boakye, Y.D.; Bekoe, E.O.; Hensel, A.; Dapaah, S.O.; Appiah, T. Review: African medicinal plants with wound healing properties. *J. Ethnopharmacol.* **2016**, *177*, 85–100. [CrossRef]
6. Kumar, B.; Vijayakumar, M.; Govindarajan, R.; Pushpangadan, P. Ethnopharmacological approaches to wound healing—Exploring medicinal plants of India. *J. Ethnopharmacol.* **2007**, *114*, 103–113. [CrossRef] [PubMed]
7. Järbrink, K.; Ni, G.; Sönnergren, H.; Schmidtchen, A.; Pang, C.; Bajpai, R.; Car, J. Prevalence and incidence of chronic wounds and related complications: A protocol for a systematic review. *Syst. Rev.* **2017**, *6*, 1–7. [CrossRef]
8. Frykberg, R.G.; Banks, J. Challenges in the Treatment of Chronic Wounds. *Adv. Wound Care* **2015**, *4*, 560–582. [CrossRef]
9. Zhao, R.; Liang, H.; Clarke, E.; Jackson, C.; Xue, M. Inflammation in chronic wounds. *Int. J. Mol. Sci.* **2016**, *17*, 2085. [CrossRef]
10. Agale, S.V. Chronic Leg Ulcers: Epidemiology, Aetiopathogenesis, and Management. *Ulcers* **2013**, *2013*, 413604. [CrossRef]
11. Ibrahim, N.I.; Wong, S.K.; Mohamed, I.N.; Mohamed, N.; Chin, K.Y.; Ima-Nirwana, S.; Shuid, A.N. Wound healing properties of selected natural products. *Int. J. Environ. Res. Public Health* **2018**, *15*, 2360. [CrossRef]
12. Marume, A.; Matope, G.; Katsande, S.; Khoza, S.; Mutingwende, I.; Mduluza, T.; Munodawafa-Taderera, T.; Ndhlala, A.R. Wound Healing Properties of Selected Plants Used in Ethnoveterinary Medicine. *Front. Pharmacol.* **2017**, *8*, 544. [CrossRef]

13. Mustoe, T. Understanding chronic wounds: A unifying hypothesis on their pathogenesis and implications for therapy. *Am. J. Surg.* **2004**, *187*, S65–S70. [CrossRef]
14. Dahal, R.H.; Chaudhary, D.K. Microbial Infections and Antimicrobial Resistance in Nepal: Current Trends and Recommendations. *Open Microbiol. J.* **2018**, *12*, 230–242. [CrossRef]
15. Guo, S.; DiPietro, L.A. Critical review in oral biology & medicine: Factors affecting wound healing. *J. Dent. Res.* **2010**, *89*, 219–229.
16. Oyebode, O.; Houreld, N.N.; Abrahamse, H. Photobiomodulation in diabetic wound healing: A review of red and near-infrared wavelength applications. *Cell Biochem. Funct.* **2021**, *39*, 596–612. [CrossRef]
17. Sabale, P.; Bhimani, B.; Prajapati, C.; Sabale, V. An overview of medicinal plants as wound healers. *J. Appl. Pharm. Sci.* **2012**, *2*, 143–150.
18. Desmet, C.M.; Préat, V.; Gallez, B. Nanomedicines and gene therapy for the delivery of growth factors to improve perfusion and oxygenation in wound healing. *Adv. Drug Deliv. Rev.* **2018**, *129*, 262–284. [CrossRef]
19. Qing, C. The molecular biology in wound healing & non-healing wound. *Chin. J. Traumatol.* **2017**, *20*, 189–193. [CrossRef]
20. Han, G.; Ceilley, R. Chronic Wound Healing: A Review of Current Management and Treatments. *Adv. Ther.* **2017**, *34*, 599–610. [CrossRef]
21. Shedoeva, A.; Leavesley, D.; Upton, Z.; Fan, C. Wound healing and the use of medicinal plants. *Evid.-Based Complementary Altern. Med.* **2019**, *2019*, 2684108. [CrossRef]
22. Kobayashi, S.D.; Malachowa, N.; DeLeo, F.R. Neutrophils and Bacterial Immune Evasion. *J. Innate Immun.* **2018**, *10*, 432–441. [CrossRef]
23. Mendonça, D.R.J.; Coutinho-Netto, J. Aspectos celulares da cicatrização. *An. Bras. Dermatol.* **2009**, *84*, 257–262. [CrossRef]
24. Tsourdi, E.; Barthel, A.; Rietzsch, H.; Reichel, A.; Bornstein, S.R. Current aspects in the pathophysiology and treatment of chronic wounds in diabetes mellitus. *BioMed Res. Int.* **2013**, *2013*, 385641. [CrossRef]
25. Harper, D.; Young, A.; McNaught, C.-E. The physiology of wound healing. *Surgery* **2014**, *32*, 445–450. [CrossRef]
26. Gonzalez, A.C.D.O.; Costa, T.F.; Andrade, Z.D.A.; Medrado, A. Wound healing—A literature review. *An. Bras. Dermatol.* **2016**, *91*, 614–620. [CrossRef]
27. Ovais, M.; Ahmad, I.; Khalil, A.T.; Mukherjee, S.; Javed, R.; Ayaz, M.; Raza, A.; Shinwari, Z.K. Wound healing applications of biogenic colloidal silver and gold nanoparticles: Recent trends and future prospects. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 4305–4318. [CrossRef]
28. Veith, A.; Henderson, K.; Spencer, A.; Sligar, A.D.; Baker, A.B. Therapeutic strategies for enhancing angiogenesis in wound healing. *Adv. Drug Deliv. Rev.* **2018**, *146*, 97–125. [CrossRef]
29. Darby, I.A.; Laverdet, B.; Bonté, F.; Desmoulière, A. Fibroblasts and myofibroblasts in wound healing. *Clin. Cosmet. Investig. Dermatol.* **2014**, *4*, 301–311.
30. Young, A.; McNaught, C.-E. The physiology of wound healing. *Surgery* **2011**, *29*, 475–479. [CrossRef]
31. Arseni, L.; Lombardi, A.; Orioli, D. From Structure to Phenotype: Impact of Collagen Alterations on Human Health. *Int. J. Mol. Sci.* **2018**, *19*, 1407. [CrossRef]
32. Akasaka, Y.; Ono, I.; Kamiya, T.; Ishikawa, Y.; Kinoshita, T.; Ishiguro, S.; Yokoo, T.; Imaizumi, R.; Inomata, N.; Fujita, K.; et al. The mechanisms underlying fibroblast apoptosis regulated by growth factors during wound healing. *J. Pathol.* **2010**, *221*, 285–299. [CrossRef]
33. Anderson, K.; Hamm, R.L. Factors that impair wound healing. *J. Am. Coll. Clin. Wound Spec.* **2014**, *4*, 84–91. [CrossRef] [PubMed]
34. Jones, S.G.; Edwards, R.; Thomas, D.W. Inflammation and Wound Healing: The Role of Bacteria in the Immuno-Regulation of Wound Healing. *Int. J. Low. Extrem. Wounds* **2004**, *3*, 201–208. [CrossRef]
35. Pfalzgraff, A.; Brandenburg, K.; Weindl, G. Antimicrobial Peptides and Their Therapeutic Potential for Bacterial Skin Infections and Wounds. *Front. Pharmacol.* **2018**, *9*, 281. [CrossRef] [PubMed]
36. Hassan, W.U.; Greiser, U.; Wang, W. Role of adipose-derived stem cells in chronic cutaneous wound healing. *Wound Repair Regen.* **2014**, *22*, 313–325. [CrossRef]
37. Sood, A.; Granick, M.; Tomaselli, N.L. Wound Dressings and Comparative Effectiveness Data. *Adv. Wound Care* **2014**, *3*, 511–529. [CrossRef] [PubMed]
38. Park, J.W.; Hwang, S.R.; Yoon, I.S. Advanced growth factor delivery systems in wound management and skin regeneration. *Molecules* **2017**, *22*, 1259. [CrossRef]
39. Li, Z.; Yu, A. Complications of negative pressure wound therapy: A mini review. *Wound Repair Regen.* **2014**, *22*, 457–461. [CrossRef]
40. Plafki, C.; Peters, P.; Almeling, M.; Welslau, W.; Busch, R. Complications and side effects of hyperbaric oxygen therapy. *Aviat. Space Environ. Med.* **2000**, *71*, 119–124.
41. Pan, S.-Y.; Zhou, S.-F.; Gao, S.-H.; Yu, Z.-L.; Zhang, S.-F.; Tang, M.-K.; Sun, J.-N.; Ma, D.-L.; Han, Y.-F.; Fong, W.-F.; et al. New Perspectives on How to Discover Drugs from Herbal Medicines: CAM's Outstanding Contribution to Modern Therapeutics. *Evid.-Based Complementary Altern. Med.* **2013**, *2013*, 627375. [CrossRef]
42. Patel, D.K. Some Traditional Medicinal Plants Useful for Boil, Burn and for Wounds Healing. *J. Biodivers. Endanger. Species* **2014**, *2*, 2–5.
43. Maroyi, A. An ethnobotanical survey of medicinal plants used by the people in Nhema communal area, Zimbabwe. *J. Ethnopharmacol.* **2011**, *136*, 347–354. [CrossRef]
44. Yahaya, E.; Cordier, W.; Steenkamp, P.; Steenkamp, V. Effect of ethnomedicinal extracts used for wound healing on cellular migration and intracellular reactive oxygen species release in SC-1 fibroblasts. *South Afr. J. Bot.* **2018**, *118*, 11–17. [CrossRef]
45. Farahpour, M.R. Medicinal plants in wound healing. *Wound Heal. Curr. Perspect.* **2019**, *2019*, 33–47. [CrossRef]
46. Annan, K.; Houghton, P.J. Antibacterial, antioxidant and fibroblast growth stimulation of aqueous extracts of Ficus asperifolia Miq. and *Gossypium arboreum* L., wound-healing plants of Ghana. *J. Ethnopharmacol.* **2008**, *119*, 141–144. [CrossRef]

47. Działo, M.; Mierziak, J.; Korzun, U.; Preisner, M.; Szopa, J.; Kulma, A. The Potential of Plant Phenolics in Prevention and Therapy of Skin Disorders. *Int. J. Mol. Sci.* **2016**, *17*, 160. [CrossRef] [PubMed]
48. Singh, B.; Sharma, S.; Dhiman, A. Acacia gum polysaccharide-based hydrogel wound dressings: Synthesis, characterization, drug delivery and biomedical properties. *Carbohydr. Polym.* **2017**, *165*, 294–303. [CrossRef]
49. Jia, Y.; Zhao, G.; Jia, J. Preliminary evaluation: The effects of Aloe ferox Miller and Aloe arborescens Miller on wound healing. *J. Ethnopharmacol.* **2008**, *120*, 181–189. [CrossRef]
50. Street, R.A.; Prinsloo, G. Commercially Important Medicinal Plants of South Africa: A Review. *J. Chem.* **2013**, *2013*, 205048. [CrossRef]
51. Pringle, N.A.; Koekemoer, T.C.; Holzer, A.; Young, C.; Venables, L.; Van De Venter, M. Potential Therapeutic Benefits of Green and Fermented Rooibos (*Aspalathus linearis*) in Dermal Wound Healing. *Planta Med.* **2018**, *84*, 645–652. [CrossRef]
52. Singhal, A.K.; Gupta, H.; Bhati, V.S. Wound healing activity of *Argyreia nervosa* leaves extract. *Int. J. Appl. Basic Med. Res.* **2011**, *1*, 36–39.
53. Chingwaru, C.; Bagar, T.; Maroyi, A.; Kapewangolo, P.T.; Chingwaru, W. Wound healing potential of selected Southern African medicinal plants: A review. *J. Herb. Med.* **2019**, *17*, 100263. [CrossRef]
54. Nair, J.J.; van Staden, J. Traditional usage, phytochemistry and pharmacology of the South African medicinal plant *Boophone disticha* (L.f.) Herb. (Amaryllidaceae). *J. Ethnopharmacol.* **2014**, *151*, 12–26. [CrossRef]
55. Adetutu, A.; Morgan, W.A.; Corcoran, O. Ethnopharmacological survey and in vitro evaluation of wound-healing plants used in South-western Nigeria. *J. Ethnopharmacol.* **2011**, *137*, 50–56. [CrossRef] [PubMed]
56. Pather, N.; Viljoen, A.M.; Kramer, B. A biochemical comparison of the in vivo effects of *Bulbine frutescens* and *Bulbine natalensis* on cutaneous wound healing. *J. Ethnopharmacol.* **2011**, *133*, 364–370. [CrossRef]
57. Nayak, B.; Anderson, M.; Pereira, L.P. Evaluation of wound-healing potential of *Catharanthus roseus* leaf extract in rats. *Fitoterapia* **2007**, *78*, 540–544. [CrossRef]
58. Van Wyk, B.-E. A review of Khoi-San and Cape Dutch medical ethnobotany. *J. Ethnopharmacol.* **2008**, *119*, 331–341. [CrossRef] [PubMed]
59. Gohil, K.; Patel, J.; Gajjar, A. Pharmacological review on *Centella asiatica*: A potential herbal cure-all. *Indian J. Pharm. Sci.* **2010**, *72*, 546. [CrossRef] [PubMed]
60. Aremu, A.; Ndhlala, A.; Fawole, O.; Light, M.; Finnie, J.; Van Staden, J. In vitro pharmacological evaluation and phenolic content of ten South African medicinal plants used as anthelmintics. *South Afr. J. Bot.* **2010**, *76*, 558–566. [CrossRef]
61. Amabeoku, G.J.; Kabatende, J. Antinociceptive and Anti-Inflammatory Activities of Leaf Methanol Extract of *Cotyledon orbiculata* L. (Crassulaceae). *Adv. Pharmacol. Sci.* **2011**, *2012*, 862625. [CrossRef]
62. Kumari, A.; Baskaran, P.; Van Staden, J. In vitro propagation and antibacterial activity in *Cotyledon orbiculata*: A valuable medicinal plant. *Plant Cell Tissue Organ Cult. (PCTOC)* **2015**, *124*, 97–104. [CrossRef]
63. Tyavambiza, C.; Elbagory, A.; Madiehe, A.; Meyer, M.; Meyer, S. The Antimicrobial and Anti-Inflammatory Effects of Silver Nanoparticles Synthesised from *Cotyledon orbiculata* Aqueous Extract. *Nanomaterials* **2021**, *11*, 1343. [CrossRef]
64. Steenkamp, V.; Mathivha, E.; Gouws, M.; van Rensburg, C.J. Studies on antibacterial, antioxidant and fibroblast growth stimulation of wound healing remedies from South Africa. *J. Ethnopharmacol.* **2004**, *95*, 353–357. [CrossRef]
65. Maroyi, A. From Traditional Usage to Pharmacological Evidence: Systematic Review of *Gunnera perpensa* L. *Evid. Based Complement. Altern. Med.* **2016**, *2016*, 1720123. [CrossRef]
66. Makgatho, M.E.; Nxumalo, W.; Raphoko, L.A. Anti-mycobacterial, -oxidative, -proliferative and -inflammatory activities of dichloromethane leaf extracts of *Gymnosporia senegalensis* (Lam.) Loes. *South Afr. J. Bot.* **2018**, *114*, 217–222. [CrossRef]
67. Fuchs, S.; Hsieh, L.T.; Saarberg, W.; Erdelmeier, C.A.J.; Wichelhaus, T.A.; Schaefer, L.; Koch, E.; Urst, R.F. Haemanthus coccineus extract and its main bioactive component narciclasine display profound anti-inflammatory activities in vitro and in vivo. *J. Cell Mol. Med.* **2015**, *19*, 1021–1032. [CrossRef]
68. Da Silva, G.; Serrano, R.; Silva, O. *Maytenus heterophylla* and *Maytenus senegalensis*, two traditional herbal medicines. *J. Nat. Sci. Biol. Med.* **2011**, *2*, 59–65. [CrossRef]
69. Nweze, E.I.; Eze, E.E. Justification for the use of *Ocimum gratissimum* L. in herbal medicine and its interaction with disc antibiotics. *BMC Complement. Altern. Med.* **2009**, *9*, 37. [CrossRef]
70. Dube, P.; Meyer, S.; Madiehe, A.; Meyer, M. Antibacterial activity of biogenic silver and gold nanoparticles synthesized from *Salvia africana-lutea* and *Sutherlandia frutescens*. *Nanotechnology* **2020**, *31*, 505607. [CrossRef]
71. Saini, N.; Singhal, M.; Srivastava, B. Evaluation of wound healing activity of *Tecomaria capensis* leaves. *Chin. J. Nat. Med.* **2012**, *10*, 138–141. [CrossRef]
72. Mongalo, N.; McGaw, L.; Segapelo, T.; Finnie, J.; Van Staden, J. Ethnobotany, phytochemistry, toxicology and pharmacological properties of Terminalia sericea Burch. ex DC. (Combretaceae)—A review. *J. Ethnopharmacol.* **2016**, *194*, 789–802. [CrossRef]
73. Eyo, J.E.; Uzoibiam, B.O.; Ogbanya, K.C.; Nnaji, T.O. Comparative evaluation of wound healing effects of ocimum gratissimum, vernonia amygdaline and zingiber officinalis extracts on incision wound model in rats. *Pharmacol. Online* **2014**, *3*, 44–50.
74. Lakkim, V.; Reddy, M.C.; Pallavali, R.R.; Reddy, K.R.; Reddy, C.V.; Bilgrami, A.L.; Lomada, D. Green synthesis of silver nanoparticles and evaluation of their antibacterial activity against multidrug-resistant bacteria and wound healing efficacy using a murine model. *Antibiotics* **2020**, *9*, 902. [CrossRef]
75. Majoumouo, M.S.; Sibuyi, N.R.S.; Tincho, M.B.; Mbekou, M.; Boyom, F.F.; Meyer, M. Enhanced Anti-Bacterial Activity of Biogenic Silver Nanoparticles Synthesized from *Terminalia mantaly* Extracts. *Int. J. Nanomed.* **2019**, *14*, 9031–9046. [CrossRef]
76. Ananth, S.; Induja, M.; Thangamathi, P.; Prabha, D.; Vinotha, K. In vitro antibacterial activity of biogenic gold nanoparticles from *Murraya koenigii* seed extract against pathogens associated with traumatic wound infections. *Int. J. Fauna Biol. Stud.* **2018**, *5*, 137–144.

77. Elbagory, A.; Meyer, M.; Cupido, C.; Hussein, A.A. Inhibition of bacteria associated with wound infection by biocompatible green synthesized gold nanoparticles from South African plant extracts. *Nanomaterials* **2017**, *7*, 417. [CrossRef]
78. Sivaranjani, V.; Philominathan, P. Synthesize of Titanium dioxide nanoparticles using *Moringa oleifera* leaves and evaluation of wound healing activity. *Wound Med.* **2016**, *12*, 1–5. [CrossRef]
79. Menon, S.; Devi, K.S.S.; Agarwal, H.; Shanmugam, V.K. Efficacy of Biogenic Selenium Nanoparticles from an Extract of Ginger towards Evaluation on Anti-Microbial and Anti-Oxidant Activities. *Colloid Interface Sci. Commun.* **2019**, *29*, 1–8. [CrossRef]
80. Vyas, J.; Rana, S. Antioxidant Activity and Biogenic Synthesis of Selenium Nanoparticles Using the Leaf Extract of Aloe Vera. *Int. J. Curr. Pharm. Res.* **2017**, *9*, 147. [CrossRef]
81. Pereira, R.F.B.; Bartolo, P.J.D.S. Traditional Therapies for Skin Wound Healing. *Adv. Wound Care* **2016**, *5*, 208–229. [CrossRef]
82. Goharshenasan, P.; Amini, S.; Atria, A.; Khorasani, G.; Abtahi, H. Topical Application of Honey on Surgical Wounds: A Randomized Clinical Trial. *Complement. Med. Res.* **2016**, *23*, 12–15. [CrossRef]
83. Edwards, J. Use of SILVERCEL® NONADHERENT on burn wounds: A case series. *Wounds UK* **2013**, *9*, 81–84.
84. Glat, P.M.; Kubat, W.D.; Hsu, J.F.; Copty, T.; Burkey, B.A.; Davis, W.; Goodwin, I. Randomized Clinical Study of SilvaSorb®Gel in Comparison to Silvadene®Silver Sulfadiazine Cream in the Management of Partial-Thickness Burns. *J. Burn. Care Res.* **2009**, *30*, 262–267. [CrossRef]
85. Hashmi, D.L.; Haith, L. The Current State of Topical Burn Treatments: A Review. *Curr. Trauma Rep.* **2019**, *5*, 160–168. [CrossRef]
86. Kuo, F.C.; Chen, B.; Lee, M.S.; Yen, S.H.; Wang, J.W. AQUACEL® Ag surgical dressing reduces surgical site infection and improves patient satisfaction in minimally invasive total knee arthroplasty: A prospective, randomized, controlled study. *BioMed Res. Int.* **2017**, *2017*, 1262108. [CrossRef]
87. Opasanon, S.; Muangman, P.; Namviriyachote, N. Clinical effectiveness of alginate silver dressing in outpatient management of partial-thickness burns. *Int. Wound J.* **2010**, *7*, 467–471. [CrossRef]
88. Paul, W.; Sharma, C.P. Alginates: Wound dressings. *Encycl. Biomed. Polym. Polym. Biomater.* **2015**, *2014*, 134–146.
89. Gunasekaran, T.; Nigusse, T.; Dhanaraju, M.D. Silver Nanoparticles as Real Topical Bullets for Wound Healing. *J. Am. Coll. Clin. Wound Spec.* **2011**, *3*, 82–96. [CrossRef]
90. Patil, A.; Mishra, V.; Thakur, S.; Riyaz, B.; Kaur, A.; Khursheed, R.; Patil, K.; Sathe, B.; Thakur, S. Nanotechnology Derived Nanotools in Biomedical Perspectives: An Update. *Curr. Nanosci.* **2018**, *15*, 137–146. [CrossRef]
91. Caster, J.; Patel, A.N.; Zhang, T.; Wang, A. Investigational nanomedicines in 2016: A review of nanotherapeutics currently undergoing clinical trials. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2016**, *9*, e1416. [CrossRef]
92. Kad, A.; Pundir, A.; Arya, S.K.; Bhardwaj, N.; Khatri, M. An Elucidative Review to Analytically Sieve the Viability of Nanomedicine Market. *J. Pharm. Innov.* **2020**, 1–17. [CrossRef] [PubMed]
93. Verbelen, J.; Hoeksema, H.; Heyneman, A.; Pirayesh, A.; Monstrey, S. Aquacel®Ag dressing versus Acticoat™ dressing in partial thickness burns: A prospective, randomized, controlled study in 100 patients. Part 1: Burn wound healing. *Burns* **2014**, *40*, 416–427. [CrossRef] [PubMed]
94. Mihai, M.M.; Dima, M.B.; Dima, B.; Holban, A.M. Nanomaterials for Wound Healing and Infection Control. *Materials* **2019**, *12*, 2176. [CrossRef]
95. Tian, J.; Wong, K.K.Y.; Ho, C.-M.; Lok, C.-N.; Yu, W.-Y.; Che, C.M.; Chiu, J.-F.; Tam, P.K.H. Topical Delivery of Silver Nanoparticles Promotes Wound Healing. *Chem. Med. Chem.* **2006**, *2*, 129–136. [CrossRef]
96. Berthet, M.; Gauthier, Y.; Lacroix, C.; Verrier, B.; Monge, C. Nanoparticle-based dressing: The future of wound treatment? *Trends Biotechnol.* **2017**, *35*, 770–784. [CrossRef]
97. Shurygina, I.A.; Shurygin, M.G. Nanoparticles in Wound Healing and Regeneration. In *Metal Nanoparticles in Pharma*; Springer International Publishing: Cham, Switzerland, 2017; pp. 21–37.
98. Arafa, M.G.; El-Kased, R.F.; Elmazar, M.M. Thermoresponsive gels containing gold nanoparticles as smart antibacterial and wound healing agents. *Sci. Rep.* **2018**, *8*, 13674. [CrossRef]
99. Lu, S.; Xia, D.; Huang, G.; Jing, H.; Wang, Y.; Gu, H. Concentration effect of gold nanoparticles on proliferation of keratinocytes. *Colloids Surf. B Biointerfaces* **2010**, *81*, 406–411. [CrossRef]
100. Gudikandula, K.; Maringanti, S.C. Synthesis of silver nanoparticles by chemical and biological methods and their antimicrobial properties. *J. Exp. Nanosci.* **2016**, *11*, 714–721. [CrossRef]
101. Aromal, S.A.; Philip, D. Green synthesis of gold nanoparticles using *Trigonella foenum-graecum* and its size-dependent catalytic activity. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2012**, *97*, 1–5. [CrossRef] [PubMed]
102. Singh, P.K.; Bhardwaj, K.; Dubey, P.; Prabhune, A. UV-assisted size sampling and antibacterial screening of *Lantana camara* leaf extract synthesized silver nanoparticles. *RSC Adv.* **2015**, *5*, 24513–24520. [CrossRef]
103. Akhtar, M.S.; Panwar, J.; Yun, Y.-S. Biogenic Synthesis of Metallic Nanoparticles by Plant Extracts. *ACS Sustain. Chem. Eng.* **2013**, *1*, 591–602. [CrossRef]
104. Jyoti, K.; Baunthiyal, M.; Singh, A. Characterization of silver nanoparticles synthesized using Urtica dioica Linn. leaves and their synergistic effects with antibiotics. *J. Radiat. Res. Appl. Sci.* **2016**, *9*, 217–227. [CrossRef]
105. Ahmadi, M.; Adibhesami, M. The effect of silver nanoparticles on wounds contaminated with Pseudomonas aeruginosa in mice: An experimental study. *Iran. J. Pharm. Res.* **2017**, *16*, 661–669.
106. Ahmed, S.; Saifullah; Ahmad, M.; Swami, B.L.; Ikram, S. Green synthesis of silver nanoparticles using *Azadirachta indica aqueous* leaf extract. *J. Radiat. Res. Appl. Sci.* **2016**, *9*, 1–7. [CrossRef]

107. Kathiravan, V.; Ravi, S.; Ashokkumar, S.; Velmurugan, S.; Elumalai, K.; Khatiwada, C.P. Green synthesis of silver nanoparticles using *Croton sparsiflorus morong* leaf extract and their antibacterial and antifungal activities. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2015**, *139*, 200–205. [CrossRef] [PubMed]
108. Bhagavathy, S.; Kancharla, S. Wound healing and angiogenesis of silver nanoparticles from *Azadirachta indica* in diabetes induced mice. *Int. J. Herb. Med.* **2016**, *4*, 24–29.
109. Chinnasamy, G.; Chandrasekharan, S.; Koh, T.W.; Bhatnagar, S. Synthesis, Characterization, Antibacterial and Wound Healing Efficacy of Silver Nanoparticles from *Azadirachta indica*. *Front. Microbiol.* **2021**, *12*, 204. [CrossRef]
110. Kumarasamyraja, D.; Swamivelmanickam, M. Evaluation of wound healing activity of biosynthesized silver nanoparticles from aqueous extract of *Cassia auriculata* L. *Int. J. Phytopharm.* **2014**, *5*, 201–209.
111. Al-Shmgani, H.; Mohammed, W.; Sulaiman, G.M.; Saadoon, A.H. Biosynthesis of silver nanoparticles from Catharanthus roseus leaf extract and assessing their antioxidant, antimicrobial, and wound-healing activities. *Artif. Cells Nanomed. Biotechnol.* **2016**, *45*, 1234–1240. [CrossRef]
112. Maghimaa, M.; Alharbi, S.A. Green synthesis of silver nanoparticles from *Curcuma longa* L. and coating on the cotton fabrics for antimicrobial applications and wound healing activity. *J. Photochem. Photobiol. B Biol.* **2020**, *204*, 111806. [CrossRef]
113. Zangeneh, M.M. Green synthesis and chemical characterization of silver nanoparticles from aqueous extract of *Falcaria vulgaris* leaves and assessment of their cytotoxicity and antioxidant, antibacterial, antifungal and cutaneous wound healing properties. *Appl. Organomet. Chem.* **2019**, *33*, e4963. [CrossRef]
114. Augustine, R.; Hasan, A.; Nath, V.K.Y.; Thomas, J.; Augustine, A.; Kalarikkal, N.; Al Moustafa, A.-E.; Thomas, S. Electrospun polyvinyl alcohol membranes incorporated with green synthesized silver nanoparticles for wound dressing applications. *J. Mater. Sci. Mater. Med.* **2018**, *29*, 163. [CrossRef]
115. Arya, G.; Kumari, R.M.; Sharma, N.; Gupta, N.; Kumar, A.; Chatterjee, S.; Nimesh, S. Catalytic, antibacterial and antibiofilm efficacy of biosynthesised silver nanoparticles using *Prosopis juliflora* leaf extract along with their wound healing potential. *J. Photochem. Photobiol. B Biol.* **2018**, *190*, 50–58. [CrossRef]
116. Raghuwanshi, N.; Kumari, P.; Srivastava, A.K.; Vashisth, P.; Yadav, T.C.; Prasad, R.; Pruthi, V. Synergistic effects of *Woodfordia fruticosa* gold nanoparticles in preventing microbial adhesion and accelerating wound healing in Wistar albino rats in vivo. *Mater. Sci. Eng. C* **2017**, *80*, 252–262. [CrossRef]
117. Chai, S.-H.; Wang, Y.; Qiao, Y.; Wang, P.; Li, Q.; Xia, C.; Ju, M. Bio fabrication of silver nanoparticles as an effective wound healing agent in the wound care after anorectal surgery. *J. Photochem. Photobiol. B Biol.* **2018**, *178*, 457–462. [CrossRef] [PubMed]
118. Fitzmaurice, S.; Sivamani, R.; Isseroff, R. Antioxidant Therapies for Wound Healing: A Clinical Guide to Currently Commercially Available Products. *Ski. Pharmacol. Physiol.* **2011**, *24*, 113–126. [CrossRef]
119. Jalilian, F.; Chahardoli, A.; Sadrjavadi, K.; Fattahi, A.; Shokoohinia, Y. Green synthesized silver nanoparticle from *Allium ampeloprasum aqueous* extract: Characterization, antioxidant activities, antibacterial and cytotoxicity effects. *Adv. Powder Technol.* **2020**, *31*, 1323–1332. [CrossRef]
120. Rajan, A.; Vilas, V.; Philip, D. Catalytic and antioxidant properties of biogenic silver nanoparticles synthesized using Areca catechu nut. *J. Mol. Liq.* **2015**, *207*, 231–236. [CrossRef]
121. Ravichandran, V.; Vasanthi, S.; Shalini, S.; Shah, S.A.A.; Harish, R. Green synthesis of silver nanoparticles using *Atrocarpus altilis* leaf extract and the study of their antimicrobial and antioxidant activity. *Mater. Lett.* **2016**, *180*, 264–267. [CrossRef]
122. Lateef, A.; Azeez, M.A.; Asafa, T.B.; Yekeen, T.A.; Akinboro, A.; Oladipo, I.C.; Azeez, L.; Ajibade, S.E.; Ojo, S.A.; Gueguim-Kana, E.B.; et al. Biogenic synthesis of silver nanoparticles using a pod extract of Cola nitida: Antibacterial and antioxidant activities and application as a paint additive. *J. Taibah Univ. Sci.* **2016**, *10*, 551–562. [CrossRef]
123. Jain, A.; Anitha, R.; Rajeshkumar, S. Antiinflammatory activity of Silver nanoparticles synthesised using Cumin oil. *Res. J. Pharm. Technol.* **2019**, *12*, 2790–2793. [CrossRef]
124. Desai, M.P.; Sangaokar, G.M.; Pawar, K.D. Kokum fruit mediated biogenic gold nanoparticles with photoluminescent, photocatalytic and antioxidant activities. *Process. Biochem.* **2018**, *70*, 188–197. [CrossRef]
125. Sangaonkar, G.M.; Pawar, K.D. Garcinia indica mediated biogenic synthesis of silver nanoparticles with antibacterial and antioxidant activities. *Colloids Surfaces B Biointerfaces* **2018**, *164*, 210–217. [CrossRef] [PubMed]
126. Bhakya, S.; Muthukrishnan, S.; Sukumaran, M.; Muthukumar, M. Biogenic synthesis of silver nanoparticles and their antioxidant and antibacterial activity. *Appl. Nanosci.* **2015**, *6*, 755–766. [CrossRef]
127. Elbagory, A.M.; Hussein, A.A.; Meyer, M. The In Vitro Immunomodulatory Effects of Gold Nanoparticles Synthesized from *Hypoxis hemerocallidea* Aqueous Extract and Hypoxoside on Macrophage and Natural Killer Cells. *Int. J. Nanomed.* **2019**, *14*, 9007–9018. [CrossRef]
128. Agarwal, H.; Shanmugam, V.K. Synthesis and optimization of zinc oxide nanoparticles using Kalanchoe pinnata towards the evaluation of its anti-inflammatory activity. *J. Drug Deliv. Sci. Technol.* **2019**, *54*, 101291. [CrossRef]
129. Alkhalaf, M.I.; Hussein, R.H.; Hamza, A. Green synthesis of silver nanoparticles by *Nigella sativa* extract alleviates diabetic neuropathy through anti-inflammatory and antioxidant effects. *Saudi J. Biol. Sci.* **2020**, *27*, 2410–2419. [CrossRef]
130. Karimzadeh, K.; Sharifi, E.; Bakhshi, N.; Ramzanpoor, M. Biogenic silver nanoparticles using *Oxalis corniculata* characterization and their clinical implications. *J. Drug Deliv. Sci. Technol.* **2019**, *54*, 101263. [CrossRef]

131. Sharifi, F.; Sharififar, F.; Sharifi, I.; Alijani, H.Q.; Khatami, M. Cytotoxicity, leishmanicidal, and antioxidant activity of biosynthesised zinc sulphide nanoparticles using *Phoenix dactylifera*. *IET Nanobiotechnol.* **2017**, *12*, 264–269. [CrossRef]
132. El-Rafie, H.M.; Hamed, M.A.-A. Antioxidant and anti-inflammatory activities of silver nanoparticles biosynthesized from aqueous leaves extracts of four Terminalia species. *Adv. Nat. Sci. Nanosci. Nanotechnol.* **2014**, *5*, 035008. [CrossRef]

CHAPTER 2.2

PUBLISHED REVIEW MANUSCRIPT: Cellular and molecular events of wound healing and the potential of silver based nanoformulations as wound healing agents

bioengineering

MDPI

Review

Cellular and Molecular Events of Wound Healing and the Potential of Silver Based Nanoformulations as Wound Healing Agents

Caroline Tyavambiza [1,2], Mervin Meyer [2] and Samantha Meyer [1,*]

[1] Department of Biomedical Sciences, Cape Peninsula University of Technology, Bellville 7535, South Africa
[2] DSI/Mintek Nanotechnology Innovation Centre, Department of Biotechnology, University of the Western Cape, Bellville 7530, South Africa
* Correspondence: meyers@cput.ac.za; Tel.: +27-21-959-6251

Abstract: Chronic wounds are a silent epidemic threatening the lives of many people worldwide. They are associated with social, health care and economic burdens and can lead to death if left untreated. The treatment of chronic wounds is very challenging as it may not be fully effective and may be associated with various adverse effects. New wound healing agents that are potentially more effective are being discovered continuously to combat these chronic wounds. These agents include silver nanoformulations which can contain nanoparticles or nanocomposites. To be effective, the discovered agents need to have good wound healing properties which will enhance their effectiveness in the different stages of wound healing. This review will focus on the process of wound healing and describe the properties of silver nanoformulations that contribute to wound healing.

Keywords: wound healing; antimicrobial; antioxidant; anti-inflammatory; silver nanoparticles; silver nanocomposites

Citation: Tyavambiza, C.; Meyer, M.; Meyer, S. Cellular and Molecular Events of Wound Healing and the Potential of Silver Based Nanoformulations as Wound Healing Agents. *Bioengineering* **2022**, *9*, 712. https://doi.org/10.3390/bioengineering9110712

Academic Editors: Hongbin Li, Feng Cheng and Guangyu Wu

Received: 4 October 2022
Accepted: 16 November 2022
Published: 19 November 2022

1. Introduction

Wound repair is a complex process that involves the coordinated interaction of various cell types, extracellular matrix molecules (ECM) and soluble mediators such as cytokines, chemokines and growth factors [1]. Wound healing is carefully divided into critical stages that happen simultaneously namely haemostasis, inflammatory, proliferation and remodelling phase [2]. These stages are equally important and failure of any one of them can disrupt the wound healing process and lead to the development of chronic wounds. New wound healing treatments are continually being discovered in a bid to find treatments that are effective in all stages of wound healing. Silver has a long history of use in the treatment of wounds. It has been widely reported to have anti-inflammatory and antimicrobial activities (even against multidrug resistant microorganisms such as methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa*) [3]. The proposed mechanisms of antimicrobial action of silver and silver nanoparticles (AgNPs) have been described in literature. Briefly, silver ions can interact with sulphur containing proteins, bind to the cell membrane and cause the disruption of the bacterial cell wall. This will eventually affect the permeability of the membrane causing leakage of metabolites, leading to cell death [4,5]. The silver ions also interfere with the electron transport system leading to increased ROS production, protein synthesis inhibition and denaturation of bacterial DNA thus inhibiting cell replication [6,7]. The anti-inflammatory activity of AgNPs are exerted through decreasing the effects of pro-inflammatory cytokines (IL 6, IL 8, IL 1beta, TNF alpha, MMP 9) [8–11], increasing the expression of anti-inflammatory cytokines (IL 10) [8] and promoting inflammatory cell death via apoptosis instead of necrosis [12]. Many silver-based compounds including colloidal silver (Silver sulfadiazine (SSD)) and nanosilver (AgNPs, nanocrystalline,

nanocomposites) have been used to make wound healing creams, bandages and hydrogels [13]. Although these silver-based formulations have improved the healing of wounds, newer formulations that are potentially more effective are continuously being discovered. Since the wound healing process is divided into different stages, it is important to understand the mechanisms of each stage in-order to promote its effectiveness. This review will describe the role of various cell types and molecules in the wound healing process. It will also review how silver containing wound healing agents including AgNPs affect these processes to promote wound healing.

2. Cells Involved in Wound Healing

2.1. Neutrophils

The wound healing process is orchestrated by different cells and signaling molecules. Neutrophils are the first cells to infiltrate the wound site after injury. The infiltration is facilitated by different signaling molecules and chemoattractants such as damage associated molecular patterns (DAMPs) released by necrotic cells, TGF-β, complement molecules (C3a and C5a), hydrogen peroxide (H_2O_2), chemokines and mediators from platelets [14,15]. CXCL8, one of the most common chemokines released by platelets α-granules, together with CXCL1, and CXCL2 play an important role in initiating inflammatory cell recruitment [16]. DAMPs released from damaged cells are known to be first signals to recruit neutrophils after tissue injury. They can directly activate neutrophils by binding to the specific Pattern Recognition Receptors (PRRs) such as toll-like receptors (TLRs) or indirectly by stimulating other cells to release neutrophil chemoattractants [17,18]. At the wound site, the recruited neutrophils also release some pro-inflammatory mediators such as TNF-α, IL-1β, IL-6 and CXCL8 which will recruit more neutrophils and other immune cells thus further promoting inflammation [19].

The main function of neutrophils is to prevent infections in the inflammatory phase by clearing the wound of any pathogens, foreign particles, and damaged tissue. They achieve this through phagocytosis, generation of an oxidative burst (due to reactive oxygen species (ROS)) and through the release of destructive proteases, antimicrobial proteins (cathepsins, defensins, lactoferrin, lysozyme) and neutrophil extracellular traps [15,20]. Neutrophils can also promote angiogenesis and the proliferation of fibroblasts and keratinocytes by increasing the expression of the cytokines; VEGF, CXCL3, IL-8, IL-1 β and MCP-1 which promote angiogenesis and proliferation [19]. After completing their task, neutrophils need to be eliminated from the wound site. These cells therefore undergo apoptosis and are subsequently phagocytized by macrophages. The elimination of neutrophils marks the transition from the inflammatory to an anti-inflammatory state [19]. Uncontrolled neutrophil migration prolongs the inflammation process leading to excessive generation of ROS and proteases. The toxic proteases and increased ROS levels degrade the ECM and damages cell membranes leading to prolonged wound healing and formation of chronic wounds [20,21].

2.2. Macrophages

Macrophages play a fundamental role in all phases of wound healing. It has been proven that the presence of macrophages promotes wound healing. Hu and colleagues (2017) reported that the increase of macrophages accelerated wound healing in both normal and diabetic mice [22]. Macrophages are initially monocytes which differentiate into macrophages after entering the tissues. These monocytes are activated and recruited to the wound site by chemoattractants (such as MCP-1) and DAMP molecules. Pro-inflammatory macrophages also referred to as M1 macrophages are involved in the inflammatory phase of wound healing while the M2 macrophages or anti-inflammatory macrophages are involved in the later stages in wound repair [23]. M1 macrophages infiltrate the wound site, 24–48 h after injury. These macrophages are highly phagocytic, they clear the wound area by phagocytosing bacteria, debris and apoptotic neutrophils (efferocytosis) [24]. M1 macrophages activates other inflammatory cells by releasing pro-inflammatory cytokines

(TNF- alpha, IL-6, and IL- beta), and growth factors (PDGF, VEGF and TGF-β1) [25]. They also release matrix metalloproteinases (MMPs) which digests the ECM, making room for infiltrating inflammatory cells and aiding migration [15]. This exacerbates efferocytosis and the pro-inflammatory state of the wound. Successful efferocytosis marks the resolution of inflammation and promotes the switch of macrophages from a pro-inflammatory to an anti-inflammatory state [26].

M2 macrophages dominate the anti-inflammatory phase of wound healing. They suppress inflammation by upregulating the expression of pro-resolutory cytokines such as IL-4, IL-10, and IL-13 [27]. They also release growth promoting growth factors including arginase 1, an important factor for effective wound repair and MMPs (MMP-12 and MMP-13) which remodel and strengthen the ECM [24]. Anti-inflammatory macrophages promote new vessel formation, angiogenesis, re-epithelialization, and the transition of fibroblasts to myofibroblasts [15,23]. Recent studies suggest that macrophages are also involved in wound resolution, the final phase of wound healing. This involves the release of anti-angiogenic factors, phagocytosis of apoptotic endothelial cells and the maturation of the epithelium [23,28].

2.3. Fibroblasts and Keratinocytes

Fibroblasts which form the major cellular component of the dermis are the key cells in the proliferation phase of wound healing. They are activated by the release of inflammatory signals mainly PDGF and TGF-beta from platelets and macrophages [14,25]. At the wound site, fibroblasts proliferate and synthesize type 1 and III collagen. They also secrete precursors for components of the ECM including hyaluronan, fibronectin, glycosaminoglycans and proteoglycans. Accumulation of the ECM is essential for the repair process as it supports cell migration [2]. Moreover fibroblasts can differentiate into myofibroblasts causing the wound to contract by contracting the wound bed and bringing the wound edges together [14].

Activated fibroblasts secrete paracrine factors such as FGF-2 and KGF which signals adjacent keratinocytes. Keratinocytes respond to these signals by producing PDGF which further stimulates fibroblasts. This kind of interaction between 2 cell types is known as cross talk [29]. Keratinocytes are the predominant cells in the epidermis, their main function in the proliferation phase is re-epithelialization. This is a crucial process which is responsible for restoring an intact epidermis after injury. Epithelialization is a multi-step process involving the proliferation, migration and differentiation of keratinocytes [30,31]. Keratinocytes also stimulate and coordinate the actions of other cell types involved in wound healing. They induce endothelial cell migration and angiogenesis via the secretion of angiogenic growth factors such as VEGF and PDGF [29,32].

3. Role of Cytokines and Growth Factors in Wound Healing

Wound healing is a complex process that involves several cell types and is controlled by various molecules, essentially cytokines and growth factors. The release of these cytokines and growth factors is programmed for each of the different phases of wound healing. Any disruption to this carefully orchestrated process may lead to the formation of non-healing chronic wounds. After injury, the pro-inflammatory cytokines, IL-1β, TNF-α and IL-6 are released to attract inflammatory cells to the wound site [21]. Chemokines such as CXCL8 (IL8), CXCL1, and CXCL2 are also released to help this process of chemotaxis [16]. At the wound site, inflammatory cells mainly macrophages release the growth factors, PDFG and TGF-beta which will recruit fibroblasts, initiating the proliferation phase. Active fibroblasts and macrophages release FGF-2 (bFGF), KGF (FGF-7), EGF, HGF, TGF-α and IGF-1 to stimulate keratinocytes which are essential in epithelization [25,29,33]. Fibroblasts, keratinocytes and macrophages further releases VEGF and PDGF to activate endothelial cells and initiate the process of angiogenesis [25]. As wound healing is a continuous and overlapping process most cytokines and growth factors function in more than 1 phase.

There are also other signaling molecules involved in wound healing. Table 1 shows a summary of the cytokines and growth factors involved in wound healing.

Table 1. Cytokines and growth factors involved in wound healing and their functions.

Growth Factor/Cytokine	Function	Reference
IL-1β, TNF-α and IL-6	Inflammation	[34]
PDGF	Chemotaxis of neutrophils and macrophages. Proliferation of fibroblasts. Induces myofibroblasts differentiation. Upregulates the production of insulin growth factor 1 (IGF-1). Stimulate angiogenesis.	[35,36]
TGF-beta	Chemotaxis of neutrophils and macrophages. Fibroblast proliferation. Myofibroblast differentiation. Stimulate re-epithelialization. Stimulate angiogenesis.	[25,37]
TGF-alpha	Stimulates proliferation and migration of keratinocytes. Induces angiogenesis.	[38,39]
bFGF (FGF-2)	Increases keratinocyte motility. Promotes the migration of fibroblasts and aids in tissue remodeling.	[2,40,41]
KGF	Stimulate keratinocyte differentiation and proliferation.	[25,39]
EGF	Promotes fibroblast and keratinocyte growth. Stimulate the proliferation and migration of keratinocytes.	[38,39]
IGF	Increases keratinocyte motility and promotes fibroblast growth.	[35]
VEGF	Increases endothelial cell migration and proliferation. Promotes angiogenesis.	[32,40]

4. Gene Expression in Wound Healing

Wound healing is orchestrated by various genes which code for different signaling molecules (cytokines, chemokines and growth factors) at the different stages of wound healing. The gene expression profile at the site of the wound varies during the different stages of wound healing. The genes are up or down regulated at different stages thus varying the influx and efflux of signaling molecules at the wound site. During the inflammatory phase, the expression of genes which code for pro-inflammatory cytokines, some chemokines and growth factors such as IL-6, TNF-a and IL-8 are upregulated while anti-inflammatory genes such as IL-10 are downregulated [42]. Pro-inflammatory genes coding for TNF activation markers, interferon (IFN) activation markers, leucocytes and macrophage markers were expressed in skin biopsies collected 2 days after wounding in basal cell carcinoma patients [43]. A study by Kubo et al. (2014) showed that the expression levels of IL-1b, IL-6, TNF-a, IFN-c and KC increased 3 h to a day after a skin burn injury in murine models [44]. This is well in the inflammatory phase which is known to take place between 1 h and 3 days after injury [45]. Moniri et al. (2018) reported that treating human dermal fibroblast cells with bacterial nanocellulose/silver (BNC/Ag) nanocomposites increased the expression of TGF-β1 from 4.8- to 11-fold at 6 and 24 h, respectively [46]. TGF-β1 is involved in almost all the processes of wound healing. It prompts the recruitment of inflammatory cells into the injury site, improves the angiogenic properties of endothelial cells, stimulates fibroblast contraction and promotes keratinocyte migration [47,48]. The presence of TGF-β1

is certainly of great importance in the wound healing process. In fact, it was stated that the chronic, nonhealing wounds often show a loss of TGF-β1 signaling [49]. However, prolonged release of this molecule can lead to hypertrophic scar formation. Hypertrophic derived fibroblasts were shown to have prolonged expression of TGF-β1 and TGF-β receptors compared to normal skin fibroblasts. In normal wound healing, the expression of TGF-β receptors decreases during the remodeling phase [49–51].

As wound healing proceeds, the expression of genes changes from a pro-inflammatory to that of a repair profile. This gene profile includes genes that promote fibroblasts and keratinocytes proliferation as well as granulation and epithelialization, these include VEGF, PDGF and FGF-2 [42]. Deonarine et al. (2007) showed that even though the proinflammatory genes were increased early (2 days) in the wound, after 4 to 8 days the profile of expressed genes changed to those of repair and angiogenesis. In this study, after day 4 and 8, the expression of type IV collagen, procollagen, integrin αv, integrin β5, MMP-2, MMP-9 and progranulin was increased [43]. These genes are involved in proliferation and maturation which occurs between approximately 4 to 21 days of wound healing. Kubo et al. (2014) reported similar results, in which the expression of VEGF, MMP-2, MMP-13 and type I collagen increased after 3 to 14 days in a skin burn injury [45]. Unlike pro-inflammatory cytokines released 3 h after wounding, IL 10 was released after 12 h and lasted up to 7 days post wounding [44]. The remodelling phase, which is the last phase of wound healing, is responsible for the development of new epithelium through restoration of tissue architecture and tissue strength [14]. In this phase, most wound healing genes are downregulated however TGF-β1 and MMPs are upregulated. TGF-β1 stimulates fibroblasts to produce type I and III collagen, while MMPs are responsible for the degradation of collagen. The activity of metalloproteinases is however tightly regulated as it might degrade essential collagen thus causing impaired healing [2,42]. Any disturbances (overexpression, under expression or prolonged expression) in the expression of genes and release of cytokines, chemokines and growth factors in wound healing will disrupt the sequence of healing which may lead to the development of chronic wounds.

5. Uses of Silver in Wound Healing

The effectiveness and the historic use of silver to control microbial infections have led to its incorporation in chronic wound treatment. Many silver containing products such as wound dressings have been developed for topical wound treatment [4]. Earliest silver preparations used for wound treatment were SSD and silver nitrate. SSD was the standard treatment for burn wounds. However, these preparations provide very high levels of the silver ion (3176 ppm), much higher than the therapeutic range (30–60 ppm) [52,53]. Due to increased silver ion levels, SSD have been reported to cause significant cellular cytotoxicity [52]. Nano formulations of silver contain lower concentrations of the silver ions and are active at lower concentrations (approximately 70 ppm), making them more suitable for wound treatment [53]. They have been shown in many instances to be more effective than SSD [54–56]. In addition to the antimicrobial activity, silver nanoformulations were reported to enhance anti-inflammatory responses, stimulate proliferation and migration of keratinocytes and fibroblasts and to improve collagen expression and formation [11,57–59]. Combining silver and nano-silver formulations with other wound treatments has been shown to have synergistic effects. Silver ions were reported to increase the activity of negative wound pressure therapy with polyurethane sponge by reducing biofilm, increasing bacterial inhibition, and reducing healing time and the cost of treatment [60–63]. Nanocrystalline wound dressing were reported to be more effective than the standard dressings. They ensure a sustained release of constant silver ions at therapeutic dose, which reduces the need to change dressings. This reduces disruption to the wound healing bed and minimizes patient discomfort and pain [7,53]. Unfortunately, silver nanoformulations do show some toxicity although to a smaller extent. Therefore, thorough toxicity studies should be done for all nanoformulations and they should be used for a limited time and only when necessary.

6. Properties of a Good Wound Healing Agent

The properties of good wound healing agents would include antimicrobial, anti-inflammatory, angiogenesis, antioxidant activity, as well as the ability to promote procollagen synthesis. They also promote the growth, differentiation and migration of fibroblasts and keratinocytes.

6.1. Antimicrobial Activity

It is essential for any wound healing agent to have antimicrobial activity. Infections particularly bacterial infections are one of the main causes of delayed wound healing. Bacteria have been more frequently isolated form wounds compared to other microorganisms such as fungi. Injury breaks the skin barrier that protects the body against microbial colonization, resulting in the exposure of the underlying tissue to various infectious organisms [64]. The skin impairment gives bacteria access to the wound site where they infiltrate, contaminate and colonize the wound. Contamination is defined as the presence of non-replicating bacteria. Contamination can lead to colonization which involves the active replication of microorganisms without eliciting an immune response [65]. Generally, all wounds are contaminated with bacteria because they provide a favorable medium for bacterial growth [66]. However, the bacterial load in the wound changes as contamination progress to colonization and finally infection. In most cases a bacterial load of more than 10^5 viable bacteria per g of wound tissue is considered as an indication of infection [67,68]. Infection occurs when the microorganisms continue to replicate to the extent of invading and damaging soft tissues thus triggering an immune response [69].

Gram positive, coagulase-negative Staphylococci (e.g., *Staphylococcus aureus*) which are usually normal skin flora are the first to enter the wound site after disruption of the intact skin barrier. This is followed by the invasion with Gram negative aerobes such as *Escherichia coli* and *Pseudomonas aeruginosa*. These aerobes reduce the oxygen levels in the wound environment promoting the invasion of anaerobes [70]. Therefore, as the wound matures into a chronic wound, anaerobes are frequently present at the wound site. Most chronic wounds contain mixed populations of microorganisms, the most common bacterial strains found at the wound site include *S. aureus*, MRSA, beta-haemolytic Streptococci, *E. coli*, *P. aeruginosa* [69,71,72].

Bacteria release toxins and proteases which can damage surrounding tissue cells in the wound area [66,73]. These toxins can also elevate the levels of pro-inflammatory cytokines thus prolonging the inflammatory phase. Due to sustained inflammation, the balance between proteases and protease inhibitors is disrupted resulting in increased levels of proteases. These proteases degrade the ECM and growth factors, hindering cell migration and prevent wound closure [65,71,74]. Pathogens can stick together and form biofilms which can be described as aggregations of microbial cells embedded in a polymeric matrix called extracellular polymeric substance (EPS). Due to the EPS layer, biofilms can evade the activity of antibiotics and host defenses thus protecting the embedded pathogens [64].

Antimicrobial activity is one of the properties for an effective wound healing agent. All wound healing agents have been shown to possess antimicrobial activities. Silver is known for its good antimicrobial activity, it has been used in the medical field since the 1900s. Due to its antimicrobial properties, its incorporation in wound healing was inevitable. Different silver-based formulations have been used in wound healing; these include SSD and newer formulations such as Aquacel, Silverlon and Acticoat [52]. Many AgNPs, although at a research level, have proved to be promising wound healing agents [75]. Various studies have proved the antimicrobial properties of these compounds.

SSD which is used in the treatment of burn wounds is a broad-spectrum antimicrobial agent active against *S. aureus*, *P. aeruginosa* and *E. coli* [4,54]. However, it does have some shortcomings, which is why wound treatments containing nanocrystalline are being developed. Nanoformulations of silver are more effective because of their smaller size which increases surface area thus increasing activity. Silver-coated polyurethane sponges were shown to reduce bacterial counts of biofilm-causing organisms such as *S. aureus*,

P. aeruginosa and MRSA [52]. Electrospun nanofibres (MADO) integrated with AgNPs were shown to be effective against the bacteria such as *S. aureus*, *P. aeruginosa* and *E. coli* [76]. Nanocomposites containing AgNPs showed significant antimicrobial activity against *S. aureus* and *E. coli* [77,78]. Many biogenic AgNPs have also been reported to have antimicrobial activity against various microorganisms [11,79–82].

6.2. Anti-Inflammatory Activity

Inflammation is the immune system's defense mechanism against various threats to the body. It is a complex process demanding the participation of both the vascular and cellular elements of the immune system. Inflammation can be either acute or chronic. Acute inflammation is short-lived, lasting for hours to days only and normally resolving after the cause of injury has been eliminated [83]. It is characterized by key features at the injury site commonly known as the cardinal signs of inflammation. These features include redness (rubor), swelling (tumor), heat (calor), pain (dolor) and loss of function (functio laesa) [84,85]. The redness and heat result from vasodilatation and increased blood flow to the inflamed site, the swelling is caused by increased vessel permeability causing leakage of fluid and plasma proteins into the interstitial area and the pain is due to an intensive sensation of harmful stimulus [84,86,87].

Any harmful stimuli to the body can incite an inflammatory response. Infection by microorganisms such as bacteria, viruses and fungi can cause inflammation [88]. Non-infectious causes of inflammation can be categorized as physical (burns, frostbites, physical injury, foreign bodies, trauma), chemical (hydrolytic enzymes, glucose, acids, toxins,) and biological [84,88]. The immune system recognizes noxious stimuli, both infectious and non-infectious, using PRR [86]. During microbial infection, PRRs identify the invading microorganisms using structures known as pathogen-associated molecular patterns (PAMPs). In non-infectious conditions, PRRs can recognize endogenous molecules released from damage cells named DAMPs [89,90]. There are different classes of PRRs including the TLRs, C-type lectin receptors (CLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs). Amongst all these, TLRs are the most extensively studied PRRs, they can bind to either PAMPs or DAMPs to initiate an inflammatory response [86,91,92].

Acute inflammation is an essential step in wound healing. It is characterized by brief vasoconstriction as an attempt to reduce blood loss after injury. Followed by vasodilation which increases the permeability of blood vessels causing leakage of plasma proteins and allowing influx of inflammatory mediators (including chemokines, cytokines, free radicals). These mediators promote leucocytes migration from the blood vessels to the site of inflammation (wound site) [93]. Neutrophils are the first cells to infiltrate the wound site followed by monocytes and lymphocytes. At the wound site monocytes differentiate into macrophages and secrete chemokines and pro-inflammatory cytokines such as IL-1, IL-6 and TNF-alpha which further promoting the inflammatory process [94]. The recruited immune cells clear off the antigens through phagocytosis and the release of ROS. Once the noxious stimulus is removed and the damage is repaired, the inflammatory response should be resolved to prevent transformation into chronic inflammation [86,93]. Mononuclear cells (macrophages, lymphocytes, and plasma cells) especially macrophages replace the pre-existing neutrophils and become the prominent cells in chronic inflammation. This type of inflammation is associated with prolonged tissue damaged, tissue granulation and fibrosis [95]. Prolonged or chronic inflammation can impair wound healing resulting in chronic wounds such as diabetic wounds [96], it can also lead to the development of various diseases such as atherosclerosis, cardiovascular diseases, and cancer [97].

Although inflammation is an essential stage of wound healing, prolonged inflammation can impair the wound healing process and lead to the development of chronic wounds [98]. Anti-inflammatory treatments can be of importance when added to wounds; they can inhibit prolonged inflammation and assist the wound to change from its inflammatory phase to the healing phase [45]. Chronic inflammation delays the proliferation

and migration of fibroblasts and keratinocytes thus delaying the process of proliferation and re-epithelization [58]. Chronic wounds are characterized by elevated levels of pro-inflammatory cytokines. Diabetic wounds become stuck in the inflammatory phase, thus disrupting the formation of growth factors and cytokines (e.g., IL10) needed for the proliferation and maturation phases [99]. Silver and AgNP based wound treatments as well as some biogenic AgNPs have been reported to possess anti-inflammatory activity. Nanocomposites made from bamboo cellulose matrix infused with AgNPs were reported to exert significant anti-inflammatory activity in mice [57,58]. The nanocomposites notability decreased the levels of the pro-inflammatory cytokines, TNFα and IL-6 and in turn shortened the wound healing time compared to the controls [57,58].

Nanocrystalline silver coated dressings were also reported to have good anti-inflammatory properties. In a study by Tian et al. (2007), AgNP grafted wound dressings reduced pro-inflammatory cytokine IL-6 levels while increasing levels of the anti-inflammatory cytokine IL-10 in 20 week old mice (BALB/C) [8]. In a porcine model of contact dermatitis, nanocrystalline silver dressings decreased inflammation by decreasing the levels of pro-inflammatory cytokines (TNFα, IL-8, MMP9), decreasing gelatinase activity and increasing apoptosis of inflammatory cells [10]. Death of inflammatory cells by necrosis causes the cells to burst and release inflammatory contents such as proteases and oxygen radicals which further increases inflammation. On the other hand, cellular death by apoptosis allows the cells to maintain the plasma membrane and inhibit the release of inflammatory contents [100]. Apoptosis of inflammatory cells therefore plays a role in resolving local inflammation in dermal wounds [12]. In another porcine study by Wright et al. (2002), nanocrystalline silver coated dressings were also effective in reducing the levels of MMPs and reducing the number of infiltrating mononuclear cells via apoptosis [12]. Controlling inflammation can therefore allow rapid wound healing as well as faster epithelization and maturation of the repaired tissue.

6.3. Antioxidant Activity

Antioxidants are known to promote the wound healing process by reducing the effects of oxidative stress in the wound [101]. They protect biological molecules such as DNA, proteins and lipids from damage by excessive amounts of ROS [102]. Although low levels of ROS and oxidative stress are important in the normal physiology of wound healing, the presence of excessive amounts can be damaging and can lead to wound healing impairment. It is therefore imperative to have a balance between the pro-oxidative and anti-oxidative systems in the body [103].

Reactive oxygen species are by-products of the normal oxygen metabolism in the body [104]. They include free radicals such as superoxide anion, and hydroxyl radical, and some non-radical molecules such as hydrogen peroxide. ROS are highly reactive and have higher oxidative potential than the molecular oxygen which is relatively stable [105]. The production of ROS is physiological, actually it is estimated that 2–5% of the total oxygen used by humans daily (around 250 g) is converted to ROS [106]. In basal levels, ROS play a significant role in wound healing as mediators of cell signaling and growth regulation [103]. Low levels of ROS are essential in all phases of wound healing namely haemostasis, inflammation, proliferation, and regeneration. During haemostasis, ROS enhances the clotting process by increasing platelet recruitment and aggregation as well as collagen induced platelet activation which promotes clot formation [106,107]. ROS also potentiates the inflammatory process by directly effecting neutrophil chemotaxis to the wound site and supporting the survival of monocytes and macrophages through the expression of monocyte colony stimulating factor-1 (CSF-1) [108]. It is also a defense mechanism that clears the wound site of any invading microbial pathogens. ROS further promotes the inflammatory response by stimulating the release of TNF-α and PDGF [107]. In wound re-epithelialization, ROS promotes fibroblast and keratinocyte proliferation and migration through mediating the signaling of FGF, EGF as well as collagenase (MMP-1) expression. ROS also stimulates angiogenesis and matrix deposition via the expression of

FGF-2 and VEGF. In addition, these oxygen species mediate conversion of fibroblasts to myofibroblasts thus aiding wound contraction [106,108,109].

ROS levels are strictly controlled and kept at the basal levels by antioxidants including enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidases and peroxiredoxins and nonenzymatic compounds such as vitamin E and glutathione [107]. Any imbalance between the production of ROS and their destruction by antioxidants leads to oxidative stress. During oxidative stress there is overexposure to ROS which have deleterious effects on wound healing [110]. Increased levels of ROS and free radicals cause severe tissue damage through lipid peroxidation, protein modification and DNA damage. These oxygen radicals break peptide bonds in the backbone of proteins thus changing the protein structure and functionality. They cause lipid peroxidation of membranes at both the cellular and the organelle level by oxidizing important protein and enzyme systems [101,103,111,112]. High levels of ROS affect wound healing in different ways; it impairs angiogenesis, inhibits the proliferation and migration of fibroblasts and prolongs inflammation [106,109]. Prolonged inflammation due to increased stimulation of neutrophil and macrophage chemotaxis and migration further increase the levels of ROS consequently increasing cellular and tissue damage. The levels of protease inhibitors are decreased thereby disrupting the protease-antiprotease balance. The excess protease uncontrollably degrades the ECM components such as collagen and proteoglycans thus delaying wound healing [106].

Nanoformulations especially those made from plants with a known history of good antioxidant activity have been shown to exhibit higher antioxidant properties which increases the wound healing activities. AgNPs synthesized using extracts of *Pongamia pinnata* showed better antioxidant potential compared to the plant extract and ascorbic acid [113]. In turn, the nanoparticles exhibited better anti-bactericidal activity and decreased wound healing time more than the extract [113]. Silver nano-colloids synthesized from antioxidant rich aqueous extracts of *Aerva javanica* had good antioxidant potential, they also showed low toxicity in both in vitro and in vivo systems [114]. After their incorporation into hydrogels, the nanoparticles incorporated hydrogels promoted rapid wound healing compared to the negative control [114]. In a study by Hajji and colleagues (2019), chitosan-PVA-silver (CS-AgNPs) nanoparticles exhibited higher antioxidant activity than the chitosan powder. Moreover, the CS-AgNPs were characterized by a low cytotoxicity effect against Chinese Hamster Ovary cells, they were also found to significantly promote wound healing [115].

6.4. Epithelization

Epithelization is an essential process in wound healing. It is defined as the formation of epithelium over a denuded surface [116]. Without re-epithelization, a wound cannot be considered healed. Impairment of the re-epithelization process will ultimately result in the development of chronic wounds. It also increases the risk of the wounds to infection and loss of fluids, which disrupts the healing process [117]. Epithelization involves the migration of epithelial cells mainly keratinocytes from one wound edge to another, forming a new tissue barrier between the wound and the outside environment [118]. To allow for migration, keratinocytes at the basal layer undergoes changes which cause them to detach from the basement membrane and neighboring cells. They also differentiate from a proliferated state to a non-proliferative state as they migrate through the granular layer [116]. Following keratinocytes migration over the wound bed, the basal keratinocytes behind the migratory cells begin to proliferate to ensure that an adequate supply of cells cover the wound [119]. Successful keratinocyte migration occurs through the fibrin and new ECM synthesized through the aid of fibroblasts. Therefore, effective epithelization occurs in the presence of fibroblasts and some endothelial cells.

During proliferation, fibroblasts accumulate at the wound site and deposit ECM components including collagen type I and III. These are used to produce a new ECM in order to replace the clot that is initially present at the wound bed [119]. ECM provides a platform for keratinocyte migration during epithelization. Some of its components particularly

collagen directly enhances keratinocyte migration. The new ECM does not resemble the ECM before injury, it is much looser thus allowing for cellular invasion [42]. Keratinocytes bind to collagen through the α1β2 integrins [117]. As there is formation of new tissue during this repair process, endothelial cells are also recruited to the wound site. They start forming new blood vessels from pre-existing ones, a process called angiogenesis [120]. Angiogenesis provides the oxygen and nutrients needed for growth of new tissue during repair. For effective wound repair, these core wound healing processes (fibroplasia, epithelization, and angiogenesis) need to occur successfully.

A good wound healing treatment should facilitate the processes of fibroplasia, angiogenesis, and epithelization. Various silver and nanosilver-based wound healing agents have been shown to enhance keratinocyte migration, fibroblast proliferation, collagen deposition and angiogenesis. Some of the functions in the proliferation and maturation phases of wound healing are included in Table 2.

Table 2. Silver based wound healing agents reported to enhance the proliferation phase of wound healing.

Wound Healing Agent	Function in Proliferation and Maturation Phase	References
Hydrogels (prepared from bamboo cellulose nanocrystals impregnated with AgNP)	Improved epithelialization. Improved collagen formation. Increased expression of collagen and growth factors (FGF, PDGF, VEGF). Improved vasculogenesis.	[57,58]
Pongamia pinnata seed extract-AgNPs loaded gel	Shortened wound healing time compared to other groups. Showed injury recuperating action, which might be because of their angiogenic and mitogenic potential	[113]
Muslin cloth coated with *Delonix elata*-AgNPs	Showed rapid wound epithelialization compared with the control.	[121]
Partially carboxymethylated cotton gauze (PCG) with AgNPs	Promotes fibroblast generation. Promotes neovascularization. Promotes formation of granulation tissue. Enhances epithelialization.	[77]
AgNP-solution-coated dressing	Increased keratinocyte proliferation and migration. Facilitates fibroblasts differentiation. Shortened wound healing time compared to other groups.	[59]
Electrospun nanofibres (MADO) integrated with AgNPs	The wound treated with MADO-AgNPs showed a complete glandular cavity, thickened epidermis, granular tissue formation, and keratinocyte restoration.	[76]

6.5. Biocompatibility

Toxicity is one of the most important factors determining the effectiveness of a wound healing agent. For effective healing, the wound healing agents being investigated should not be toxic to the normal cells surrounding the wound. The treatments need to have selective toxicity, being toxic only to microorganisms and damaged cells and not to normal healthy cells. It has been reported that damage to keratinocytes and fibroblasts impairs wound healing. Most common wound therapies such as negative pressure wound therapy, hyperbaric oxygen and administration of growth factors can be toxic to tissues and are therefore associated with complications such as bleeding and pneumothorax [13,75]. They are also very costly therefore silver-based therapies which are more cost-effective were investigated as alternative therapies. Silver-based therapies have promising wound healing properties, they have a

broad antimicrobial spectrum, are good anti-inflammatory agents and were shown to shorten the wound healing time [13]. However, despite their popularity, some silver based wound therapies were also shown to be toxic to normal cells. Toxicity of silver-based products has been attributed to the release of the silver ions. Silver diffuses through the wound into the skin cells and causes a grey skin discoloration known as argyria [13,122–124]. In some cases, the prolonged use of silver products may also cause epithelial and fibroblast toxicity especially if high concentrations of silver are used [7,125–127]. Systemic toxicity is however rare because silver ions can be neutralized by anions in body fluids [75]. The more silver ions are released, the higher the toxic effects they present. For example, SSD, the widely used treatment for burn wounds has been found to release high levels of silver (3.176 ppm) into the wounds [52]. It has been reported that SSD has high local toxicity and can also cause bone marrow toxicity because of the presence of propylene glycol. Newer silver formulations in the form of nanoparticles and nanocrystalline are being used for wound healing. These formulations were found to be more effective while being less toxic as they use much lower concentrations of silver. AgNPs and nanocrystalline silver were shown to be more effective and less toxic than SSD and silver nitrate in various studies [52,54–56]. The toxicity of AgNPs has however been reported, AgNPs exert their toxicity through ROS generation, protein oxidation and membrane damage [128]. Nanoparticle toxicity is determined by their characteristics with the most important one being the size [129]. In most cases smaller nanoparticles are more toxic than the larger ones, smaller nanoparticles can easily enter into cells and pass-through biological membranes thus having a higher chance of causing cellular toxicity [128,130]. It is therefore important to thoroughly investigate the toxic effects of these nanoformulations before using them for wound treatments. The toxicity of Ag and AgNPs has been extensively covered in other review articles [52,123,128–133].

7. Incorporation of the Silver Formulations in Wound Dressings

The incorporation of wound healing agents into wound dressing can greatly facilitate wound healing. An ideal wound dressing is one that protects the wound from external contamination, is biocompatible and semipermeable to oxygen and water [134]. All this keeps the wound moist, which facilitates epithelialization in wound healing. An ideal wound dressing should also be cost effective, non-toxic and hypoallergenic, it should be nonadherent and can be easily removed without trauma [4]. Traditionally used wound dressings such as the gauze, cotton and wool cannot sufficiently provide wound care requirements, they can cause drying out of wounds and lead to trauma upon removal [4,58]. Therefore, newer dressings made from biodegradable materials such as chitosan, hyaluronic acid, collagen, silicon, cellulose, and gelatin are being used. These new materials do not only preserve the wound environment, they also release bioactive compounds in the wound and aid in the process of healing [124,134]. Incorporation of silver and its nanoformulations in wound dressings have been sown to enhance wound healing. Silver and nano-silver wound dressings have been reported to have; significant antimicrobial activity, decrease inflammation, improved collagen expression and increased epithelial cell differentiation, proliferation and migration [8,57,58,76–78]. All these properties allow the silver wound dressings to improve the wound healing process leading to faster wound healing compared to normal dressings. Silver wound dressings have also been associated with reduced pain and anxiety as well as lower treatment costs thus improving patients' lifestyle [55,135]. Many other silver and nano-silver formulations are still to be explored. Good examples are the biogenic AgNPs synthesized using medicinal plants known to possess wound healing activities. A few of these, including AgNPs synthesized from *Cotyledon orbiculata* [11], and *Garcinia indica* [136] have been reported in literature.

8. Conclusions

Wound healing is a complex process that is carefully divided into critical stages which happen simultaneously. It is a delicate process in which failure of any one of them can disrupt the whole wound healing process and lead to the development of chronic

wounds. Wound healing agents need to have good wound healing properties which will enhance their effectiveness in the different stages of wound repair. Good wound healing agents have properties such as antimicrobial, anti-inflammatory, angiogenic and antioxidant activities. They can also promote the growth, differentiation and migration of fibroblasts and keratinocytes. Different silver nanoformulations were shown to enhance wound healing at different stages. Most of the formulations were effective in more than one stage of healing however none of them was effective in all the wound healing stages. Combination therapy in which one or more compounds are combined in the treatment of a wound, or where different compounds are applied to the wound based on the stage of wound healing may be our answer to effectively treat chronic wounds. It is therefore of great importance to continuously research and develop different therapies and combination therapies that can successfully combat problematic chronic wounds.

Author Contributions: Conceptualization, S.M. and M.M.; writing—original draft preparation, C.T.; writing—review and editing, C.T., S.M. and M.M.; supervision, S.M. and M.M.; project administration, C.T.; funding acquisition, M.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the South African National Research Foundation (grant number 114598 and 106642) and the DSI/MINTEK Nanotechnology Innovation Centre (NIC).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AgNP	silver nanoparticle
CLR	C-type lectin receptor
CSF-1	colony stimulating factor-1
DAMP	damage associated molecular pattern
ECM	extracellular matrix molecules
EGF	epidermal growth factor
FGF	fibroblast growth factor
IFN	interferon
IGF	insulin growth factor 1
IL	interleukin
KGF	Keratinocyte growth factor
MMP	matrix metalloproteinase
NLR	NOD-like receptor
PAMP	pathogen-associated molecular pattern
PDGF	platelet-derived growth factor
PRR	pattern recognition receptor
RLR	Retinoic acid-inducible gene (RIG)-I-like receptor
ROS	reactive oxygen species
SOD	superoxide dismutase
SSD	silver sulfadiazine
TGF	Transforming growth factor
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor

References

1. Dehkordi, A.N.; Babaheydari, F.M.; Chehelgerdi, M.; Raeisi Dehkordi, S. Skin Tissue Engineering: Wound Healing Based on Stem-Cell-Based Therapeutic Strategies. *Stem Cell Res. Ther.* **2019**, *10*, 1–20. [CrossRef]
2. Enoch, S.; Leaper, D.J. Basic Science of Wound Healing. *Surgery* **2008**, *26*, 31–37. [CrossRef]
3. Rai, M.K.; Deshmukh, S.D.; Ingle, A.P.; Gade, A.K. Silver Nanoparticles: The Powerful Nanoweapon against Multidrug-Resistant Bacteria. *J. Appl. Microbiol.* **2012**, *112*, 841–852. [CrossRef] [PubMed]
4. Konop, M.; Damps, T.; Misicka, A.; Rudnicka, L. Certain Aspects of Silver and Silver Nanoparticles in Wound Care: A Minirev. *EBSCOhost* **2016**, *2016*, 7614753. [CrossRef]
5. Naik, K.; Kowshik, M. The Silver Lining: Towards the Responsible and Limited Usage of Silver. *J. Appl. Microbiol.* **2017**, *123*, 1068–1087. [CrossRef]
6. Yin, I.X.; Zhang, J.; Zhao, I.S.; Mei, M.L.; Li, Q.; Chu, C.H. The Antibacterial Mechanism of Silver Nanoparticles and Its Application in Dentistry. *Int. J. Nanomed.* **2020**, *15*, 2555–2562. [CrossRef]
7. Fong, J.; Wood, F. Nanocrystalline Silver Dressings in Wound Management: A Review. *Int. J. Nanomed.* **2006**, *1*, 441–449. [CrossRef]
8. Tian, J.; Wong, K.K.Y.; Ho, C.; Lok, C.; Yu, W.; Che, C.; Chiu, J.; Tam, P.K.H. Topical Delivery of Silver Nanoparticles Promotes Wound Healing. *ChemMedChem* **2007**, *2*, 129–136. [CrossRef]
9. Wong, K.K.Y.; Cheung, S.O.F.; Huang, L.; Niu, J.; Tao, C.; Ho, C.-M.; Che, C.-M.; Tam, P.K.H. Further Evidence of the Anti-Inflammatory Effects of Silver. *ChemMedChem* **2009**, *4*, 1129–1135. [CrossRef]
10. Nadworny, P.L.; Wang, J.; Tredget, E.E.; Burrell, R.E. Anti-Inflammatory Activity of Nanocrystalline Silver in a Porcine Contact Dermatitis Model. *Nanomedicine* **2008**, *4*, 241–251. [CrossRef]
11. Tyavambiza, C.; Elbagory, A.M.; Madiehe, A.M.; Meyer, M.; Meyer, S.; Elaissari, A. The Antimicrobial and Anti-Inflammatory Effects of Silver Nanoparticles Synthesised from Cotyledon Orbiculata Aqueous Extract. *Nanomaterials* **2021**, *11*, 1343. [CrossRef]
12. Wright, J.B.; Lam, K.A.N.; Buret, A.G.; Olson, M.E.; Burrell, R.E. Early Healing Events in a Porcine Model of Contaminated Wounds: Effects of Nanocrystalline Silver on Matrix Metalloproteinases, Cell Apoptosis, and Healing. *Wound Repair Regen.* **2002**, *10*, 141–151. [CrossRef]
13. Nqakala, Z.B.; Sibuyi, N.R.S.; Fadaka, A.O.; Meyer, M.; Onani, M.O.; Madiehe, A.M. Advances in Nanotechnology towards Development of Silver Nanoparticle-Based Wound-Healing Agents. *Int. J. Mol. Sci.* **2021**, *22*, 11272. [CrossRef]
14. Velnar, T.; Bailey, T.; Smrkolj, V. The Wound Healing Process: An Overview of the Cellular and Molecular Mechanisms. *J. Int. Med. Res.* **2009**, *37*, 1528–1542. [CrossRef]
15. Rodrigues, M.; Kosaric, N.; Bonham, C.A.; Gurtner, G.C. Wound Healing: A Cellular Perspective. *Physiol. Rev.* **2018**, *99*, 665–706. [CrossRef]
16. Ridiandries, A.; Tan, J.T.M.; Bursill, C.A. The Role of Chemokines in Wound Healing. *Int. J. Mol. Sci.* **2018**, *19*, 3217. [CrossRef]
17. Pittman, K.; Kubes, P. Damage-Associated Molecular Patterns Control Neutrophil Recruitment. *J. Innate Immun.* **2013**, *5*, 315–323. [CrossRef]
18. De Oliveira, S.; Rosowski, E.E.; Huttenlocher, A. Neutrophil Migration in Infection and Wound Repair: Going Forward in Reverse. *Nat. Rev. Immunol.* **2016**, *16*, 378–391. [CrossRef]
19. Ellis, S.; Lin, E.J.; Tartar, D. Immunology of Wound Healing. *Curr. Dermatol. Rep.* **2018**, *7*, 350–358. [CrossRef]
20. Serra, M.B.; Barroso, W.A.; da Silva, N.N.; Silva, S.D.N.; Borges, A.C.R.; Abreu, I.C.; da Rocha Borges, M.O. From Inflammation to Current and Alternative Therapies Involved in Wound Healing. *Int. J. Inflamm.* **2017**, *2017*, 3406215. [CrossRef]
21. Beserra, F.P.; Gushiken, L.F.S.; Hussni, M.F.; Pellizzon, C.H. Regulatory Mechanisms and Chemical Signaling of Mediators Involved in the Inflammatory Phase of Cutaneous Wound Healing. In *Wound Healing—Current Perspectives*; IntechOpen: London, UK, 2018; ISBN 978-1-78985-538-8.
22. Hu, M.S.; Walmsley, G.G.; Barnes, L.A.; Weiskopf, K.; Rennert, R.C.; Duscher, D.; Januszyk, M.; Maan, Z.N.; Hong, W.X.; Cheung, A.T.M.; et al. Delivery of Monocyte Lineage Cells in a Biomimetic Scaffold Enhances Tissue Repair. *JCI Insight* **2017**, *2*, e96260. [CrossRef] [PubMed]
23. Dipietro, L.A.; Wilgus, T.A.; Koh, T.J. Macrophages in Healing Wounds: Paradoxes and Paradigms. *Int. J. Mol. Sci.* **2021**, *22*, 950. [CrossRef] [PubMed]
24. Krzyszczyk, P.; Schloss, R.; Palmer, A.; Berthiaume, F. The Role of Macrophages in Acute and Chronic Wound Healing and Interventions to Promote Pro-Wound Healing Phenotypes. *Front. Physiol.* **2018**, *9*, 419. [CrossRef] [PubMed]
25. Yussof, S.J.M.; Omar, E.; Pai, D.R.; Sood, S. Cellular Events and Biomarkers of Wound Healing. *Indian J. Plast. Surg.* **2012**, *45*, 220. [CrossRef] [PubMed]
26. Khanna, S.; Biswas, S.; Shang, Y.; Collard, E.; Azad, A.; Kauh, C.; Bhasker, V.; Gordillo, G.M.; Sen, C.K.; Roy, S. Macrophage Dysfunction Impairs Resolution of Inflammation in the Wounds of Diabetic Mice. *PLoS ONE* **2010**, *5*, e9539. [CrossRef]
27. Wilkinson, H.N.; Hardman, M.J. Wound Healing: Cellular Mechanisms and Pathological Outcomes. *Open Biol.* **2020**, *10*, 1–14. [CrossRef]
28. Michaeli, S.; Dakwar, V.; Weidenfeld, K.; Granski, O.; Gilon, O.; Schif-Zuck, S.; Mamchur, A.; Shams, I.; Barkan, D. Soluble Mediators Produced by Pro-Resolving Macrophages Inhibit Angiogenesis. *Front. Immunol.* **2018**, *9*, 1–12. [CrossRef]
29. Wojtowicz, A.M.; Oliveira, S.; Carlson, M.W.; Zawadzka, A.; Rousseau, C.F.; Baksh, D. The Importance of Both Fibroblasts and Keratinocytes in a Bilayered Living Cellular Construct Used in Wound Healing. *Wound Repair Regen.* **2014**, *22*, 246. [CrossRef]

30. Piipponen, M.; Li, D.; Landén, N.X. The Immune Functions of Keratinocytes in Skin Wound Healing. *Int. J. Mol. Sci.* **2020**, *21*, 8790. [CrossRef]
31. Mi, B.; Liu, J.; Liu, G.; Zhou, W.; Liu, Y.; Hu, L.; Xiong, L.; Ye, S.; Wu, Y. Icariin Promotes Wound Healing by Enhancing the Migration and Proliferation of Keratinocytes via the AKT and ERK Signaling Pathway. *Int. J. Mol. Med.* **2018**, *42*, 831–838. [CrossRef]
32. Barrientos, S.; Stojadinovic, O.; Golinko, M.S.; Brem, H.; Tomic-Canic, M. PERSPECTIVE ARTICLE: Growth Factors and Cytokines in Wound Healing. *Wound Repair Regen.* **2008**, *16*, 585–601. [CrossRef]
33. Seeger, M.A.; Paller, A.S. The Roles of Growth Factors in Keratinocyte Migration. *Adv. Wound Care* **2015**, *4*, 213. [CrossRef]
34. Barrientos, S.; Brem, H.; Stojadinovic, O.; Tomic-Canic, M. Clinical Application of Growth Factors and Cytokines in Wound Healing. *Wound Repair Regen. Off. Publ. Wound Health Soc. Eur. Tissue Repair Soc.* **2014**, *22*, 569. [CrossRef]
35. Krishnaswami, S.; Ly, Q.P.; Rothman, V.L.; Tuszynski, G.P. Thrombospondin-1 Promotes Proliferative Healing through Stabilization of PDGF. *J. Surg. Res.* **2002**, *107*, 124–130. [CrossRef]
36. Lin, H.; Chen, B.; Sun, W.; Zhao, W.; Zhao, Y.; Dai, J. The Effect of Collagen-Targeting Platelet-Derived Growth Factor on Cellularization and Vascularization of Collagen Scaffolds. *Biomaterials* **2006**, *27*, 5708–5714. [CrossRef]
37. Werner, S.; Grose, R. Regulation of Wound Healing by Growth Factors and Cytokines. *Physiol. Rev.* **2003**, *83*, 835–870. [CrossRef]
38. Traversa, B.; Sussman, G. The Role of Growth Factors, Cytokines and Proteases in Wound Management. *Prim. Intent.* **2001**, *9*, 161–167.
39. Park, J.W.; Hwang, S.R.; Yoon, I.S. Advanced Growth Factor Delivery Systems in Wound Management and Skin Regeneration. *Molecules* **2017**, *22*, 1259. [CrossRef]
40. Di Vita, G.; Patti, R.; D'Agostino, P.; Caruso, G.; Arcara, M.; Buscemi, S.; Bonventre, S.; Ferlazzo, V.; Arcoleo, F.; Cillari, E. Cytokines and Growth Factors in Wound Drainage Fluid from Patients Undergoing Incisional Hernia Repair. *Wound Repair Regen.* **2006**, *14*, 259–264. [CrossRef]
41. Sogabe, Y.; Abe, M.; Yokoyama, Y.; Ishikawa, O. Basic Fibroblast Growth Factor Stimulates Human Keratinocyte Motility by Rac Activation. *Wound Repair Regen.* **2006**, *14*, 457–462. [CrossRef]
42. Diller, R.B.; Tabor, A.J. The Role of the Extracellular Matrix (ECM) in Wound Healing: A Review. *Biomimetics* **2022**, *7*, 87. [CrossRef] [PubMed]
43. Deonarine, K.; Panelli, M.C.; Stashower, M.E.; Jin, P.; Smith, K.; Slade, H.B.; Norwood, C.; Wang, E.; Marincola, F.M.; Stroncek, D.F. Gene Expression Profiling of Cutaneous Wound Healing. *J. Transl. Med.* **2007**, *5*, 1–11. [CrossRef] [PubMed]
44. Kubo, H.; Hayashi, T.; Ago, K.; Ago, M.; Kanekura, T.; Ogata, M. Temporal Expression of Wound Healing-Related Genes in Skin Burn Injury. *Leg. Med.* **2014**, *16*, 8–13. [CrossRef] [PubMed]
45. Lande'n, N.X.; Li, D.; Stahle, M. Transition from Inflammation to Proliferation: A Critical Step during Wound Healing. *Cell. Mol. Life Sci.* **2016**, *73*, 3861–3885. [CrossRef] [PubMed]
46. Moniri, M.; Moghaddam, A.B.; Azizi, S.; Rahim, R.A.; Zuhainis, S.W.; Navaderi, M.; Mohamad, R. In Vitro Molecular Study of Wound Healing Using Biosynthesized Bacteria Nanocellulose/ Silver Nanocomposite Assisted by Bioinformatics Databases. *Int. J. Nanomed.* **2018**, *13*, 5097–5112. [CrossRef]
47. Pakyari, M.; Farrokhi, A.; Maharlooei, M.K.; Ghahary, A. Critical Role of Transforming Growth Factor Beta in Different Phases of Wound Healing. *Adv. Wound Care* **2013**, *2*, 215. [CrossRef]
48. Evrard, S.M.; Daudigier, C.; Mauge, L.; Israël-Biet, D.; Guerin, C.L.; Bieche, I.; Kovacic, J.C.; Fischer, A.M.; Gaussem, P.; Smadja, D.M. The Profibrotic Cytokine Transforming Growth Factor-B1 Increases Endothelial Progenitor Cell Angiogenic Properties. *J. Thromb. Haemost.* **2012**, *10*, 670–679. [CrossRef]
49. Penn, J.W.; Grobbelaar, A.O.; Rolfe, K.J. The Role of the TGF-β Family in Wound Healing, Burns and Scarring: A Review. *Int. J. Burn. Trauma* **2012**, *2*, 18–28.
50. Wang, R.; Ghahary, A.; Shen, Q.; Scott, P.G.; Roy, K.; Tredget, E.E. Hypertrophic Scar Tissues and Fibroblasts Produce More Transforming Growth Factor-B1 MRNA and Protein than Normal Skin and Cells. *Wound Repair Regen.* **2000**, *8*, 128–137. [CrossRef]
51. Schmid, P.; Itin, P.; Cherry, G.; Bi, C.; Cox, D.A. Enhanced Expression of Transforming Growth Factor-Beta Type I and Type II Receptors in Wound Granulation Tissue and Hypertrophic Scar. *Am. J. Pathol.* **1998**, *152*, 485.
52. Khansa, I.; Schoenbrunner, A.R.; Kraft, C.T.; Janis, J.E. Silver in Wound Care—Friend or Foe?:A Comprehensive Review. *Plast. Reconsructive Surg. Glob. Open* **2019**, *7*, 1–10. [CrossRef]
53. Murphy, P.S.; Evans, G.R.D. Advances in Wound Healing: A Review of Current Wound Healing Products. *Plast. Surg. Int.* **2012**, *2012*, 1–8. [CrossRef]
54. Opasanon, S.; Muangman, P.; Namviriyachote, N. Clinical Effectiveness of Alginate Silver Dressing in Outpatient Management of Partial-Thickness Burns. *Int. Wound J.* **2010**, *7*, 467–471. [CrossRef]
55. Muangman, P.; Pundee, C.; Opasanon, S.; Muangman, S. A Prospective, Randomized Trial of Silver Containing Hydrofiber Dressing versus for the Treatment of Partial Thickness Burns. *Int. Wound J.* **2010**, *7*, 271–276. [CrossRef]
56. Nam, G.; Rangasamy, S.; Purushothaman, B.; Song, J.M. The Application of Bactericidal Silver Nanoparticles in Wound Treatment. *Nanomater. Nanotechnol.* **2015**, *5*, 1–14. [CrossRef]
57. Singla, R.; Soni, S.; Markand, P.; Kumari, A.; Mahesh, S.; Patial, V.; Padwad, Y.S.; Yadav, S.K. In Situ Functionalized Nanobiocomposites Dressings of Bamboo Cellulose Nanocrystals and Silver Nanoparticles for Accelerated Wound Healing. *Carbohydr. Polym.* **2017**, *155*, 152–162. [CrossRef]

58. Singla, R.; Soni, S.; Patial, V.; Markand, P.; Kumari, A.; Mahesh, S.; Padwad, Y.S.; Yadav, S. International Journal of Biological Macromolecules In Vivo Diabetic Wound Healing Potential of Nanobiocomposites Containing Bamboo Cellulose Nanocrystals Impregnated with Silver Nanoparticles. *Int. J. Biol. Macromol.* **2017**, *105*, 45–55. [CrossRef]
59. Liu, X.; Lee, P.; Ho, C.; Lui, V.C.H.; Chen, Y.; Che, C.; Tam, P.K.H.; Wong, K.K.Y. Silver Nanoparticles Mediate Differential Responses in Keratinocytes and Fibroblasts during Skin Wound Healing. *ChemMedChem* **2010**, *5*, 468–475. [CrossRef]
60. Ngo, Q.D.; Vickery, K.; Deva, A.K. The Effect of Topical Negative Pressure on Wound Biofilms Using an in Vitro Wound Model. *Wound Repair Regen.* **2011**, *20*, 83–90. [CrossRef]
61. Ellenrieder, M.; Redanz, S.; Bader, R.; Mittelmeier, W.; Podbielski, A. Influence of Antimicrobial Coatings of Vacuum-Assisted Closure Dressings on Methicillin-Resistant Staphylococcus Aureus Growth Kinetics: An in vitro study. *Surg. Infect.* **2015**, *16*, 139–145. [CrossRef]
62. Sachsenmaier, S.; Peschel, A.; Ipach, I.; Kluba, T. Antibacterial Potency of V.A.C. GranuFoam Silver 1 Dressing. *Injury* **2013**, *44*, 1363–1367. [CrossRef] [PubMed]
63. Karr, J.C.; De Mola, F.L.; Pham, T.; Tooke, L. Wound Healing and Cost-Saving Benefits of Combining Negative-Pressure Wound Therapy with Silver. *Adv. Skin Wound Care* **2013**, *26*, 562–565. [CrossRef] [PubMed]
64. Sarheed, O.; Ahmed, A.; Shouqair, D.; Boateng, J. Antimicrobial Dressings for Improving Wound Healing. In *Wound Healing: New Insights into Ancient Challenges*; IntechOpen: London, UK, 2016. [CrossRef]
65. Guo, S.; DiPietro, L.A. Factors Affecting Wound Healing. *J. Dent. Res.* **2010**, *89*, 219. [CrossRef] [PubMed]
66. Moore, K.; Gray, D. Using PHMB Antimicrobial to Prevent Wound Infection. *Wounds Int.* **2007**, *3*, 96–102.
67. Leaper, D.J. Silver Dressings: Their Role in Wound Management. *Int. Wound J.* **2006**, *3*, 282–294. [CrossRef] [PubMed]
68. Sen, R.K.; Murthy, N.R.S.; Gill, S.S.; Nagi, O.N. Bacterial Load in Tissues and Its Predictive Value for Infection in Open Fractures. *J. Orthop. Surg.* **2000**, *8*, 1–5. [CrossRef]
69. Negut, I.; Grumezescu, V.; Grumezescu, A.M. Treatment Strategies for Infected Wounds. *Molecules* **2018**, *23*, 2392. [CrossRef]
70. Daeschlein, G. Antimicrobial and Antiseptic Strategies in Wound Management. *Int. Wound J.* **2013**, *10*, 9–14. [CrossRef]
71. Zhao, R.; Liang, H.; Clarke, E.; Jackson, C.; Xue, M. Inflammation in Chronic Wounds. *Int. J. Mol. Sci.* **2016**, *17*, 2085. [CrossRef]
72. Bowler, P.G.; Duerden, B.I.; Armstrong, D.G. Wound Microbiology and Associated Approaches to Wound Management. *Clin. Microbiol. Rev.* **2001**, *14*, 244–269. [CrossRef]
73. Houghton, P.J.; Hylands, P.J.; Mensah, A.Y.; Hensel, A.; Deters, A.M. In Vitro Tests and Ethnopharmacological Investigations: Wound Healing as an Example. *J. Ethnopharmacol.* **2005**, *100*, 100–107. [CrossRef]
74. Mustoe, T. Understanding Chronic Wounds: A Unifying Hypothesis on Their Pathogenesis and Implications for Therapy. *Am. J. Surg.* **2004**, *187*, S65–S70. [CrossRef]
75. Tyavambiza, C.; Dube, P.; Goboza, M.; Meyer, S.; Madiehe, A.M.; Meyer, M. Wound Healing Activities and Potential of Selected African Medicinal Plants and Their Synthesized Biogenic Nanoparticles. *Plants* **2021**, *10*, 2635. [CrossRef]
76. Ghavaminejad, A.; Unnithan, A.R.; Ramachandra, A.; Sasikala, K.; Samarikhalaj, M.; Thomas, R.G.; Jeong, Y.Y.; Nasseri, S.; Murugesan, P.; Wu, D.; et al. Mussel-Inspired Electrospun Nano Fi Bers Functionalized with Size- Controlled Silver Nanoparticles for Wound Dressing Application. *Appl. Mater. Interface* **2015**, *7*, 12176–12183. [CrossRef]
77. Wu, T.; Lu, F.; Wen, Q.; Yu, K.; Lu, B.; Rong, B.; Dai, F.; Lan, G. Novel Strategy for Obtaining Uniformly Dispersed Silver Nanoparticles on Soluble Cotton Wound Dressing through Carboxymethylation and In-Situ Reduction: Antimicrobial Activity and Histological Assessment in Animal Model. *Cellulose* **2018**, *25*, 5361–5376. [CrossRef]
78. Zhang, H.; Peng, M.; Cheng, T.; Qiu, L. Biomaterials Silver Nanoparticles-Doped Collagen—Alginate Antimicrobial Biocomposite as Potential Wound Dressing. *J. Mater. Sci.* **2018**, *53*, 14944–14952. [CrossRef]
79. Dube, P.; Meyer, S.; Madiehe, A.; Meyer, M. Antibacterial Activity of Biogenic Silver and Gold Nanoparticles Synthesized from Salvia Africana-Lutea and Sutherlandia Frutescens. *Nanotechnology* **2020**, *31*, 505607. [CrossRef]
80. Elbagory, A.; Meyer, M.; Cupido, C.; Hussein, A.A. Inhibition of Bacteria Associated with Wound Infection by Biocompatible Green Synthesized Gold Nanoparticles from South African Plant Extracts. *Nanomaterials* **2017**, *7*, 417. [CrossRef]
81. Majoumouo, M.S.; Sibuyi, N.R.S.; Tincho, M.B.; Mbekou, M.; Boyom, F.F.; Meyer, M. Enhanced Anti-Bacterial Activity of Biogenic Silver Nanoparticles Synthesized from Terminalia Mantaly Extracts. *Int. J. Nanomed.* **2019**, *14*, 9031–9046. [CrossRef]
82. Mmola, M.; Roes-Hill, M.; Durrell, K.; Bolton, J.; Sibuyi, N.; Meyer, M.; Beukes, D.; Antunes, E. Enhanced Antimicrobial and Anticancer Activity of Silver and Gold Nanoparticles Synthesised Using Sargassum Incisifolium Aqueous Extracts. *Molecules* **2016**, *21*, 1633. [CrossRef]
83. Furman, D.; Campisi, J.; Verdin, E.; Carrera-Bastos, P.; Targ, S.; Franceschi, C.; Ferrucci, L.; Gilroy, D.W.; Fasano, A.; Miller, G.W.; et al. Chronic Inflammation in the Etiology of Disease across the Life Span. *Nature Medicine* **2019**, *25*, 1822–1832. [CrossRef] [PubMed]
84. Stankov, S. V Definition of Inflammation, Causes of Inflammation and Possible Anti-Inflammatory Strategies. *Open Inflamm. J.* **2012**, *5*, 1–9. [CrossRef]
85. Medzhitov, R. Inflammation 2010: New Adventures of an Old Flame. *Cell* **2010**, *140*, 771–776. [CrossRef] [PubMed]
86. Takeuchi, O.; Akira, S. Pattern Recognition Receptors and Inflammation. *Cell* **2010**, *140*, 805–820. [CrossRef] [PubMed]
87. Pober, J.S.; Sessa, W.C. Inflammation and the Blood Microvascular System. *Cold Spring Harb. Perspect. Biol.* **2015**, *7*, 1–11. [CrossRef]

88. Chen, L.; Deng, H.; Cui, H.; Fang, J.; Zuo, Z.; Deng, J.; Li, Y.; Wang, X.; Zhao, L. Inflammatory Responses and Inflammation-Associated Diseases in Organs. *Oncotarget* **2018**, *9*, 7204–7218. [CrossRef]
89. Sharma, S.K.; Naidu, G. The Role of Danger-Associated Molecular Patterns (DAMPs) in Trauma and Infections. *J. Thorac. Dis.* **2016**, *8*, 1406–1409. [CrossRef]
90. Roh, J.S.; Sohn, D.H. Damage-Associated Molecular Patterns in Inflammatory Diseases. *Immune Netw.* **2018**, *18*, e27. [CrossRef]
91. Amarante-Mendes, G.P.; Adjemian, S.; Branco, L.M.; Zanetti, L.C.; Weinlich, R.; Bortoluci, K.R. Pattern Recognition Receptors and the Host Cell Death Molecular Machinery. *Front. Immunol.* **2018**, *9*, 2379. [CrossRef]
92. Martin, S.J. Cell Death and Inflammation: The Case for IL-1 Family Cytokines as the Canonical DAMPs of the Immune System. *FEBS J.* **2016**, *283*, 2599–2615. [CrossRef]
93. Sugimoto, M.A.; Vago, J.P.; Perretti, M.; Teixeira, M.M. Mediators of the Resolution of the Inflammatory Response. *Trends Immunol.* **2019**, *40*, 212–227. [CrossRef]
94. Koh, T.J.; DiPietro, L.A. Inflammation and Wound Healing: The Role of the Macrophage. *Expert Rev. Mol. Med.* **2011**, *13*, e23. [CrossRef]
95. Abdulkhaleq, L.A.; Assi, M.A.; Abdullah, R.; Zamri-Saad, M.; Taufiq-Yap, Y.H.; Hezmee, M.N.M. The Crucial Roles of Inflammatory Mediators in Inflammation: A Review. *Vet. World* **2018**, *11*, 627–635. [CrossRef]
96. Eming, S.A.; Martin, P.; Tomic-Canic, M. Wound Repair and Regeneration: Mechanisms, Signaling, and Translation. *Sci. Transl. Med.* **2014**, *6*, 265sr6. [CrossRef]
97. Sugimoto, M.A.; Sousa, L.P.; Pinho, V.; Perretti, M.; Teixeira, M.M. Resolution of Inflammation: What Controls Its Onset? *Front. Immunol.* **2016**, *7*, 160. [CrossRef]
98. Shedoeva, A.; Leavesley, D.; Upton, Z.; Fan, C. Wound Healing and the Use of Medicinal Plants. *Evid. -Based Complement. Altern. Med.* **2019**, *2019*, 2684108. [CrossRef]
99. Tsourdi, E.; Barthel, A.; Rietzsch, H.; Reichel, A.; Bornstein, S.R. Current Aspects in the Pathophysiology and Treatment of Chronic Wounds in Diabetes Mellitus. *BioMed Res. Int.* **2013**, *2013*, 385641. [CrossRef]
100. Fink, S.L.; Cookson, B.T. MINIREVIEW Apoptosis, Pyroptosis, and Necrosis: Mechanistic Description of Dead and Dying Eukaryotic Cells. *Infect. Immun.* **2005**, *73*, 1907–1916. [CrossRef]
101. Süntar, I.; Akkol, E.K.; Nahar, L.; Sarker, S.D. Wound Healing and Antioxidant Properties: Do They Coexist in Plants? *Free Radic. Antioxid.* **2012**, *2*, 1. [CrossRef]
102. Barku, V.Y. Wound Healing: Contributions from Plant Secondary Metabolite Antioxidants. In *Wound Healing Current Perspectives*; IntechOpen: London, UK, 2019. [CrossRef]
103. Fitzmaurice, S.D.; Sivamani, R.K.; Isseroff, R.R. Antioxidant Therapies for Wound Healing: A Clinical Guide to Currently Commercially Available Products. *Ski. Pharmacol. Physiol.* **2011**, *24*, 113–126. [CrossRef]
104. Ozcan, A.; Ogun, M. Biochemistry of Reactive Oxygen and Nitrogen Species. In *Basic Principles and Clinical Significance of Oxidative Stress*; IntechOpen: London, UK, 2015. [CrossRef]
105. Rex, J.R.S.; Muthukumar, N.M.; Selvakumar, P.M. Phytochemicals as a Potential Source for Anti-Microbial, Anti-Oxidant and Wound Healing—A Review. *MOJ Bioorganic Org. Chem.* **2018**, *2*, 61–70. [CrossRef]
106. Soneja, A.; Drews, M.; Malinski, T. Role of Nitric Oxide, Nitroxidative and Oxidative Stress in Wound Healing. *Pharmacol. Rep.* **2005**, *57*, 108–119. [PubMed]
107. Sanchez, M.C.; Lancel, S.; Boulanger, E.; Neviere, R. Targeting Oxidative Stress and Mitochondrial Dysfunction in the Treatment of Impaired Wound Healing: A Systematic Review. *Antioxidants* **2018**, *7*, 98. [CrossRef] [PubMed]
108. Sen, C.K. The General Case for Redox Control of Wound Repair. *Wound Repair Regen.* **2003**, *11*, 431–438. [CrossRef] [PubMed]
109. Kurahashi, T.; Fujii, J. Roles of Antioxidative Enzymes in Wound Healing. *J. Dev. Biol.* **2015**, *3*, 57–70. [CrossRef]
110. Poljsak, B.; Šuput, D.; Milisav, I. Achieving the Balance between ROS and Antioxidants: When to Use the Synthetic Antioxidants. *Oxid. Med. Cell. Longev.* **2013**, *2013*, 956792. [CrossRef]
111. Barku, V. Wound Healing: Contributions from Medicinal Plants and Their Phytoconstituents. *Annu. Res. Rev. Biol.* **2018**, *26*, 1–14. [CrossRef]
112. Shetty, B.S. Wound Healing and Indigenous Drugs: Role as Antioxidants: A Review. *Res. Rev. J. Med. Health Sci.* **2013**, *2*, 5–16.
113. Paul, M.; Londe, Y.V. Pongamia Pinnata Seed Extract—Mediated Green Synthesis of Silver Nanoparticles: Preparation, Formulation and Evaluation of Bactericidal and Wound Healing Potential. *Appl. Organomet. Chem.* **2018**, *33*, e4624. [CrossRef]
114. Hashmi, U.M.; Khan, F.; Khalid, N.; Shahid, A.A.; Javed, A.; Alam, T.; Jalal, N.; Hayat, Q.M.; Abbas, R.S.; Janjua, A.H. Hydrogels Incorporated with Silver Nanocolloids Prepared from Antioxidant Rich Aerva Javanica as Disruptive Agents against Burn Wound Infections. *Colloids Surf. A* **2017**, *529*, 475–486. [CrossRef]
115. Hajji, S.; Khedir, B.S.; Hamza-mnif, I.; Hamdi, M.; Jedidi, I.; Kallel, R.; Boufi, S.; Nasri, M. BBA—General Subjects Biomedical Potential of Chitosan-Silver Nanoparticles with Special Reference to Antioxidant, Antibacterial, Hemolytic and in Vivo Cutaneous Wound Healing Effects. *BBA Gen. Subj.* **2019**, *1863*, 241–254. [CrossRef]
116. Pastar, I.; Stojadinovic, O.; Yin, N.C.; Ramirez, H.; Nusbaum, A.G.; Sawaya, A.; Patel, S.B.; Khalid, L.; Isseroff, R.R.; Tomic-canic, M. Epithelialization in Wound Healing: A Comprehensive Review. *Adv. Wound Care* **2014**, *3*, 445–464. [CrossRef]
117. Tan, S.T.; Dosan, R. Lessons From Epithelialization: The Reason Behind Moist Wound Environment. *Open Dermatol. J.* **2019**, *13*, 34–40. [CrossRef]

118. Ben Amar, M.; Wu, M. Re-Epithelialization: Advancing Epithelium Frontier during Wound Healing. *J. R. Soc. Interface* **2014**, *11*, 1–7. [CrossRef]
119. Woodley, D.T.; Wysong, A.; Declerck, B.; Chen, M.; Li, W. Keratinocyte Migration and a Hypothetical New Role for Extracellular Heat Shock Protein 90 Alpha in Orchestrating Skin Wound Healing. *Adv. Wound Care* **2015**, *4*, 203–212. [CrossRef]
120. Nagaraja, S.; Chen, L.; Dipietro, L.A.; Reifman, J.; Mitrophanov, A.Y.; Finley, S.D. Predictive Approach Identifies Molecular Targets and Interventions to Restore Angiogenesis in Wounds with Delayed. *Health Front. Physiol.* **2019**, *10*, 1–17. [CrossRef]
121. Chai, S.; Wang, Y.; Qiao, Y.; Wang, P.; Li, Q.; Xia, C.; Ju, M. Journal of Photochemistry & Photobiology, B: Biology Bio Fabrication of Silver Nanoparticles as an e Ff Ective Wound Healing Agent in the Wound Care after Anorectal Surgery. *J. Photochem. Photobiol. B Biol.* **2018**, *178*, 457–462. [CrossRef]
122. Lansdown, A.B.G. A Pharmacological and Toxicological Profile of Silver as an Antimicrobial Agent in Medical Devices. *Adv. Pharmacol. Sci.* **2010**, *2010*, 910686. [CrossRef]
123. Ferdous, Z.; Nemmar, A. Health Impact of Silver Nanoparticles: A Review of the Biodistribution and Toxicity Following Various Routes of Exposure. *Int. J. Mol. Sci.* **2020**, *21*, 2375. [CrossRef]
124. Kalantari, K.; Mostafavi, E.; Afifi, A.M.; Izadiyan, Z.; Jahangirian, H.; Rafiee-Moghaddam, R.; Webster, T.J. Wound Dressings Functionalized with Silver Nanoparticles: Promises and Pitfalls. *Nanoscale* **2020**, *12*, 2268–2291. [CrossRef]
125. Frazer, J.F.; Cuttle, L.; Kempf, M.; Kimble, R.M. Cytotoxicity of topical antimicrobial agents used in burn wounds in Australasia. *ANZ J. Surg.* **2004**, *74*, 139–142. [CrossRef] [PubMed]
126. Poon, V.K.M.; Burd, A. In Vitro Cytotoxity of Silver: Implication for Clinical Wound Care. *Burns* **2004**, *30*, 140–147. [CrossRef]
127. Talapko, J.; Matijevi, T.; Juzbaši, M.; Antolovi, A.; Škrlec, I. Antibacterial Activity of Silver and Its Application in Dentistry, Cardiology and Dermatology. *Microorganisms* **2020**, *8*, 1400. [CrossRef] [PubMed]
128. Tortella, G.R.; Rubilar, O.; Durán, N.; Diez, M.C.; Martínez, M.; Parada, J.; Seabra, A.B. Silver Nanoparticles: Toxicity in Model Organisms as an Overview of Its Hazard for Human Health and the Environment. *J. Hazard. Mater.* **2020**, *390*, 121974. [CrossRef] [PubMed]
129. Akter, M.; Sikder, M.T.; Rahman, M.M.; Ullah, A.K.M.A.; Hossain, K.F.B.; Banik, S.; Hosokawa, T.; Saito, T.; Kurasaki, M. A Systematic Review on Silver Nanoparticles-Induced Cytotoxicity: Physicochemical Properties and Perspectives. *J. Adv. Res.* **2018**, *9*, 1–16. [CrossRef]
130. Zhang, T.; Wang, L.; Chen, Q.; Chen, C. Cytotoxic Potential of Silver Nanoparticles. *Yonsei Med. J.* **2014**, *55*, 283–291. [CrossRef]
131. Dos Santos, C.A.; Seckler, M.M.; Ingle, A.P.; Gupta, I.; Galdiero, S.; Galdiero, M.; Gade, A.; Rai, M. Silver Nanoparticles: Therapeutical Uses, Toxicity, and Safety Issues. *J. Pharm. Sci.* **2014**, *103*, 1931–1944. [CrossRef]
132. McShan, D.; Ray, P.C.; Yu, H. Molecular Toxicity Mechanism of Nanosilver. *J. Food Drug Anal.* **2014**, *22*, 116–127. [CrossRef]
133. Yu, Z.; Li, Q.; Wang, J.; Yu, Y.; Wang, Y.; Zhou, Q.; Li, P. Reactive Oxygen Species-Related Nanoparticle Toxicity in the Biomedical Field. *Nanoscale Res. Lett.* **2020**, *15*, 115. [CrossRef]
134. Naskar, A.; Kim, K. Recent Advances in Nanomaterial-Based Wound-Healing Therapeutics. *Pharmaceutics* **2020**, *12*, 499. [CrossRef]
135. Mabrouk, A.; Boughdadi, N.S.; Helal, H.A.; Zaki, B.M.; Maher, A. Moist Occlusive Dressing (Aquacel W Ag) versus Moist Open Dressing (MEBO W) in the Management of Partial-Thickness Facial Burns: A Comparative Study in Ain Shams University. *Burns* **2011**, *38*, 396–403. [CrossRef]
136. Sangaonkar, G.M.; Pawar, K.D. Colloids and Surfaces B: Biointerfaces Garcinia Indica Mediated Biogenic Synthesis of Silver Nanoparticles with Antibacterial and Antioxidant Activities. *Colloids Surf. B Biointerfaces* **2018**, *164*, 210–217. [CrossRef]

Introduction to Chapter 3 and 4

Fresh *C. orbiculata* plants were purchased from the Van Der Berg Garden Village nursery in Stellenbosch, South Africa. In preparation of the water extract, the leaves (300 g) were cut from the whole plant, washed with distilled water, and cut into small pieces. They were then blended and macerated in 600 mL of distilled water overnight. Thereafter, the extract was filtered using Whatman filter paper No.1, followed by microfiltration using 0.45 µm filters. The extract was then concentrated by freeze drying and stored at 4 ◦C until use. Silver nanoparticles were synthesised from the *C. orbiculata* water extract using a method by Tyavambiza et al. (2021). Briefly 1 ml of the *C. orbiculata* water extract (3 mg/ml) was mixed with 5 ml of $AgNO_3$ at 3 mM. The mixture was incubated for 2 hours at 70 °C. After incubation, the solution changed from colourless to brown, indicative of AgNPs. Characterisation was done using the UV-vis spectrophotometer and the High Resolution Transmission Electron Microscopy (HR-TEM). As shown in the figure below, the synthesised nanoparticles were similar to those synthesised by Tyavambiza et al. 2021.

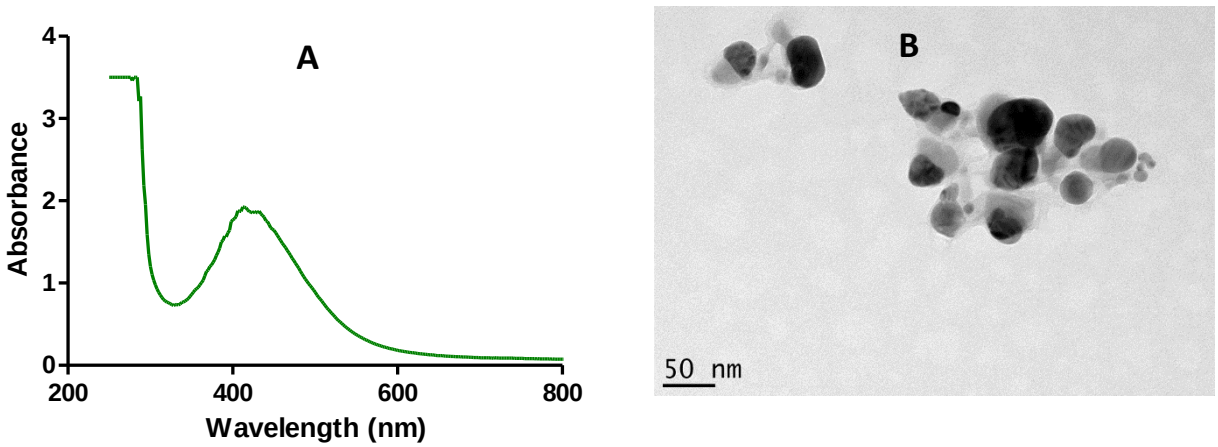


Figure 1. Characterization of *C. orbiculata* synthesized silver nanoparticles using (A) UV-vis spectroscopy and (B) HR-TEM. The synthesized nanoparticles had a maximum absorbance at a wavelength of 416 nm and were approximately 40-60 nm in size.

The *Cotyledon*–AgNPs were previously reported to possess antimicrobial and anti-inflammatory properties. In this study the wound healing properties and cytotoxic effects of the *Cotyledon*-AgNPs were investigated in Chapter 3 and 4 respectively.

Reference

Tyavambiza, C., Elbagory, A.M., Madiehe, A.M., Meyer, M. & Meyer, S. 2021. The

Antimicrobial and Anti-Inflammatory Effects of Silver Nanoparticles Synthesised from Cotyledon orbiculata Aqueous Extract. *Nanomaterials*. 11(5): 1343. https://doi.org/10.3390/nano11051343.

CHAPTER 3

PUBLISHED MANUSCRIPT: The antioxidant and in vitro wound healing activity of *Cotyledon orbiculata* aqueous extract and the synthesized biogenic silver nanoparticles

International Journal of
Molecular Sciences

MDPI

Article

The Antioxidant and In Vitro Wound Healing Activity of *Cotyledon orbiculata* Aqueous Extract and the Synthesized Biogenic Silver Nanoparticles

Caroline Tyavambiza [1,2], Mervin Meyer [2], Adedoja Dorcas Wusu [2], Abram Madimabe Madiehe [2,3] and Samantha Meyer [1,*]

[1] Department of Biomedical Sciences, Cape Peninsula University of Technology, P.O. Box 1906, Bellville 7535, South Africa
[2] DSI/Mintek Nanotechnology Innovation Centre, Department of Biotechnology, University of the Western Cape, Private Bag X17, Bellville 7530, South Africa
[3] Nanobiotechnology Research Group, Department of Biotechnology, University of the Western Cape, Private Bag X17, Bellville 7530, South Africa
* Correspondence: meyers@cput.ac.za; Tel.: +27-21-959-6251

Abstract: The synthesis of silver nanoparticles using biogenic methods, particularly plants, has led to the discovery of several effective nanoparticles. In many instances, plant-derived silver nanoparticles have been shown to have more activity than the plant extract which was used to synthesize the nanoparticles. Silver nanoparticles have been successfully synthesized using the medicinal plant, *Cotyledon orbiculata.* This is a shrub found in the Western Cape province of South Africa. It has a long history of use in traditional medicine in the treatment of wounds and skin infections. The *C. orbiculata* synthesized silver nanoparticles (*Cotyledon*-AgNPs) were reported to have good antimicrobial and anti-inflammatory activities; however, their wound-healing properties have not been determined. This study aimed to determine the wound healing activity of *Cotyledon*-AgNPs using the scratch assay. Gene expression studies were also done to determine the nanoparticles' mechanism of action. The *Cotyledon*-AgNPs showed good antioxidant, growth-promoting and cell migration properties. Gene expression studies showed that the *C. orbiculata* water extract and *Cotyledon*-AgNPs promoted wound healing by upregulating genes involved in cell proliferation, migration and growth while downregulating pro-inflammatory genes. This confirms, for the first time that a water extract of *C. orbiculata* and silver nanoparticles synthesized from this extract are good wound-healing agents.

Keywords: silver nanoparticles; wound healing; gene expression; cell proliferation; cell migration

Citation: Tyavambiza, C.; Meyer, M.; Wusu, A.D.; Madiehe, A.M.; Meyer, S. The Antioxidant and In Vitro Wound Healing Activity of *Cotyledon orbiculata* Aqueous Extract and the Synthesized Biogenic Silver Nanoparticles. *Int. J. Mol. Sci.* **2022**, *23*, 16094. https://doi.org/10.3390/ijms232416094

Academic Editors: Simona Martinotti and Elia Ranzato

Received: 6 November 2022
Accepted: 15 December 2022
Published: 17 December 2022

1. Introduction

Green nanotechnology is receiving much attention in all scientific research areas worldwide. Its popularity can be attributed to the ability to synthesize biocompatible nanomaterials using simple, eco-friendly, and cost-effective methods. This field of nanotechnology involves the use of biological materials such as plants and microorganisms in nanomaterial synthesis. This significantly reduces energy costs, the use of toxic chemicals and some expensive materials and instruments which are common with the chemical and physical methods [1]. Recently, green nanotechnology has been applied in the form of nanoparticles, nanocomposites, hydrogels and nanofibers for the development of products that are used for wound treatment [2]. Metallic nanoparticles containing silver, gold and zinc are the most studied due to their unique properties most notably their antibacterial activity [3]. In addition to its antibacterial activity, silver promotes wound healing and has been used in wound dressings for the treatment of different types of wounds including burns and wounds [4]. Biogenic silver nanoparticles (AgNPs) have become popular antimicrobial, anti-inflammatory, and good wound healing agents [5]. The wound-healing potential of biogenic nanoparticles in both in vitro and in vivo models has been reported.

South Africa is a richly biodiverse country with a strong history of traditional medicinal practice. It hosts around 30,000 flowering plant species and accounts for almost 10% of the world's higher plant species [6,7]. *C. orbiculata* is one example of a plant that is used in traditional medicine in South Africa. It is used in home gardens as an ornamental plant, but its leaves are also sold as herbal medicines in the informal herbal medicine markets in different provinces of South Africa [8]. *C. orbiculata* commonly known as pig's ear is a succulent shrub widely distributed in the Western Cape province of South Africa. It has a long history of use in traditional medicine. *C. orbiculata* has been used for the treatment of skin rashes, abscesses, sores and wounds, inflammation, boils, acne, corns and warts, earache, toothache, epilepsy and syphilis [9,10]. Studies have reported that *C. orbiculata* possesses various biological activities such as antibacterial, antifungal, anticonvulsant, antinociceptive, anti-inflammatory, anthelmintic, anticancer and antioxidant activities [8,11,12]. The traditional uses and the reported biological activities suggest that *C. orbiculata* might be a good wound-healing agent. A study by Mhlongo et al. 2022, showed that the ethyl acetate extract of *C. orbiculata* has wound-healing activity [13]. The extract, however, did not show anti-inflammatory and collagen production activities.

Wound healing is a complex and highly regulated physiological process that aims to restore the anatomical structure and function of injured tissue [14]. This process involves various growth factors and cytokines including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF), tumor necrosis factor-alpha (TNF-α) and interleukin 1 (IL-1) [15–17]. Wound healing is divided into four integrated phases namely hemostasis, inflammation, proliferation, and tissue remodeling. Hemostasis, comprising vascular constriction, platelet aggregation and coagulation is intended to stop bleeding caused by the injury [17,18]. The inflammatory phase involves the infiltration of the wound site by neutrophils and monocytes to fight off any microorganisms that might have invaded the wound [18]. The proliferation phase is characterized by tissue reconstruction through the formation of granulation tissue (involving fibroblasts), angiogenesis (involving endothelial cells), wound contraction and epithelialization (involving keratinocytes) [15,18,19]. Fibroblasts and myofibroblasts cause the wound to contract by pulling the wound edges together. Epithelial cells will migrate from the wound edges, divide and proliferate along the surface of the granulation tissue until these cells form a complete sheet that covers the wound [20]. In the last phase of remodeling, there is collagen remodeling and vascular maturation, eventually leading to the flattening and strengthening of the scar [21].

Many factors can affect the wound-healing process resulting in chronic, non-healing wounds. Chronic wounds are those wounds that fail to heal in a timely and orderly manner [22]. These wounds are a silent epidemic and a global threat to the health of many individuals worldwide [23]. They affect the physical and mental health of patients, they lead to severe disability, multiple organ failure and eventually the death of the patient [24]. The discovery of effective wound healing agents to combat chronic wounds is, therefore, of paramount importance.

We demonstrated previously that AgNPs synthesized using extracts of *C. orbiculata* (*Cotyledon*-AgNPs) have exceptional antibacterial and anti-inflammatory activities [25]. Because *C. orbiculata* is also used in traditional medicine for the treatment of wounds, we set out to investigate the wound-healing properties of extracts of *C. orbiculata* as well as *Cotyledon*-AgNPs.

2. Results and Discussion

2.1. Phytochemical Analysis and Antioxidant Studies

The presence of phytochemicals such as polyphenols, flavanols, tannins and flavonols in *C. orbiculata* water extract and *Cotyledon*-AgNPs was investigated. As shown in Table 1, polyphenols and flavanols were shown to be 15.07 ± 1.31 mg GAE/g and 0.71 ± 0.04 mg CE/g in *Cotyledon*-AgNPs as compared to those of the extract which were 37.39 ± 0.18 mg GAE/g and 4.34 ± 0.65 mg CE/g, respectively. This may suggest that some of the polyphenols and flavanols

were involved in the synthesis of the AgNPs. The synthesis of AgNPs using polyphenols has been previously reported [26–28]. Flavonols on the other hand were higher in *Cotyledon*-AgNPs as compared to the extract with the values of 15.64 ± 0.70 and 1.67 ± 0.16 mg QE/g, respectively. This could mean that flavonols are the main phytochemicals involved in the synthesis of *Cotyledon*-AgNPs. Flavonoids can form stable complexes by chelating metal ions through their multiple hydroxyl (–OH) groups. They are responsible for the production of nanoparticles through reduction as well as nanoparticle growth, nucleation, and stabilization [29,30]. Several studies obtained similar results where the synthesized AgNPs exhibited more flavonols than the plant extract [31–33]. Although the involvement of proteins, carbohydrates, terpenoids and alkaloids has been reported in metallic nanoparticle synthesis [34], polyphenols and flavonols are the major phytochemicals involved in this process [29,34]. Phytochemicals act as both the reducing and stabilizing agents in metallic nanoparticle synthesis [35].

Table 1. Phytochemical analysis and antioxidant studies.

Treatment (1 mg/mL)	*C. orbiculata* Water Extract	*Cotyledon*-AgNPs
Polyphenols (mg GAE/g)	37.39 ± 0.18	15.07 ± 1.31
Flavonols (mg QE/g)	1.67 ± 0.16	15.64 ± 0.70
Flavanol and tannins (mg CE/g)	4.34 ± 0.65	0.71 ± 0.04
FRAP µmol AAE/g	258.84 ± 2.75	127.28 ± 10.9
ABTS µmol TE/g	91.14 ± 0.04	134.54 ± 20.59

Phenolic compounds, including flavonols, are not only reducing agents and metal chelators, but they are also good free radical scavengers. Due to their reducing and radical scavenger properties, phenols and flavanols have been reported to possess significant antioxidant activities. The antioxidant activities of the *C. orbiculata* water extract and *Cotyledon*-AgNPs were determined using the Ferric Reducing Antioxidant Power (FRAP) and 2′-Azino-Bis-3-Ethylbenzotiazolin-6- Sulfonic Acid (ABTS) assays. The FRAP assay directly measures the amount of antioxidants in a sample by measuring their ferric-reducing activity [36]. However, other antioxidant assays such as ABTS operate on free radical inhibition via hydrogen atom transfer [37]. As shown in Table 1, both the *C. orbiculata* water extract and the *Cotyledon*-AgNPs exhibited some antioxidant activity. In the FRAP assay, the *C. orbiculata* water extract showed more reducing activity than the *Cotyledon*-AgNPs. The *Cotyledon*-AgNPs were able to reduce approximately half the amount of ferric ions compared to the *C. orbiculata* water extract. This finding corresponds to the total polyphenol content (TPC) results, where approximately half of the polyphenols present in the extracts were used in the synthesis of the *Cotyledon*-AgNPs. Literature reports that the amount of iron reduced in the FRAP assay can be correlated with the amount of antioxidants present [38,39]. This could, therefore, mean that of all the phytochemicals present on the *Cotyledon*-AgNPs, polyphenols are the ones responsible for the ferric-reducing activity in the FRAP assay. On the other hand, the ABTS assay presented the *Cotyledon*-AgNPs as more active than the *C. orbiculata* water extract with values of 134.54 ± 20.59 and 91.14 ± 0.04, respectively. The difference in these results is indicative of the different mechanisms between the FRAP and the ABTS assays. Flavanols, phenolic acids, flavonols and tannins may be responsible for the reported bioactivities of *C. orbiculata*, as they have been shown to possess antimicrobial and anti-inflammatory activities [40]. These phytochemicals may as well be attributed to the traditional use of the plant. Increased antioxidant activity of AgNPs has been shown to increase their wound healing capabilities [41]. High ROS levels interfere with fibroblast proliferation, and oxidative stress causes damage to cell membranes, proteins and lipids resulting in delayed wound healing [41]. Therefore, antioxidant substances that can scavenge ROS and maintain non-toxic ROS levels in the wound tissues could improve healing [42]. Several biomaterials with antioxidant properties have been developed. In a study by Castro et al. 2015, an antioxidant dressing was prepared and tested using in vitro and in vivo methods. The dressing was shown to have protective effects by reducing ROS levels in fibroblasts; it also regulated the expression

of inflammation related genes. In the in vivo studies, the antioxidant dressing promoted faster wound healing compared to the control [43].

2.2. Cell Viability and Growth Rate

The cell viability and growth-promoting effects of the *C. orbiculata* water extract and *Cotyledon*-AgNPs were assessed on KMST, HaCaT and CHO cells using the WST-1 assay. The viability of cells treated with the *C. orbiculata* water extract and the *Cotyledon*-AgNPs were both dose and time-dependent. The *Cotyledon*-AgNPs reduced the viability of HaCaT and CHO cells at higher concentrations ($\geq$5 µg/mL for HaCaT and $\geq$20 µg/mL for CHO); however, they were non-toxic to KMST-6 cells at all the concentrations tested. These results correspond to the findings by Zanette et al. 2011, where AgNPs toxicity to HaCaT cells increased with increasing incubation time and concentration [44]. Interestingly, at lower concentrations, the *Cotyledon*-AgNPs increased the rate of cell growth (Figure 1). At concentrations between 0.1–2.5 µg/mL, *Cotyledon*-AgNPs increased the growth of KMST-6 and CHO cells at both 24 and 72 h. The *Cotyledon*-AgNPs also increased the growth rate of HaCaT cells at concentrations between 0.1 and 2.5 and 0.1 and 1.3 µg/mL at 24 and 72 h, respectively.

The *C. orbiculata* water extract did not reduce the viability of HaCaT and CHO cells at both 24 and 72 h incubation. However, they reduced the viability of KMST-6 cells at concentrations higher than 240 µg/mL. The *C. orbiculata* water extract promoted cell growth at most of the concentrations tested especially the lower concentrations. Unlike the *Cotyledon*-AgNPs which decreased cell growth as incubation time increased, the *C. orbiculata* water extract actually increased the growth of cells with increasing incubation time. The concentrations of 2.5 µg/mL (*Cotyledon*-AgNPs) and 15 µg/mL (*C. orbiculata* water extract) were used for the scratch wound healing assay as these showed a stable trend amongst the different cell lines.

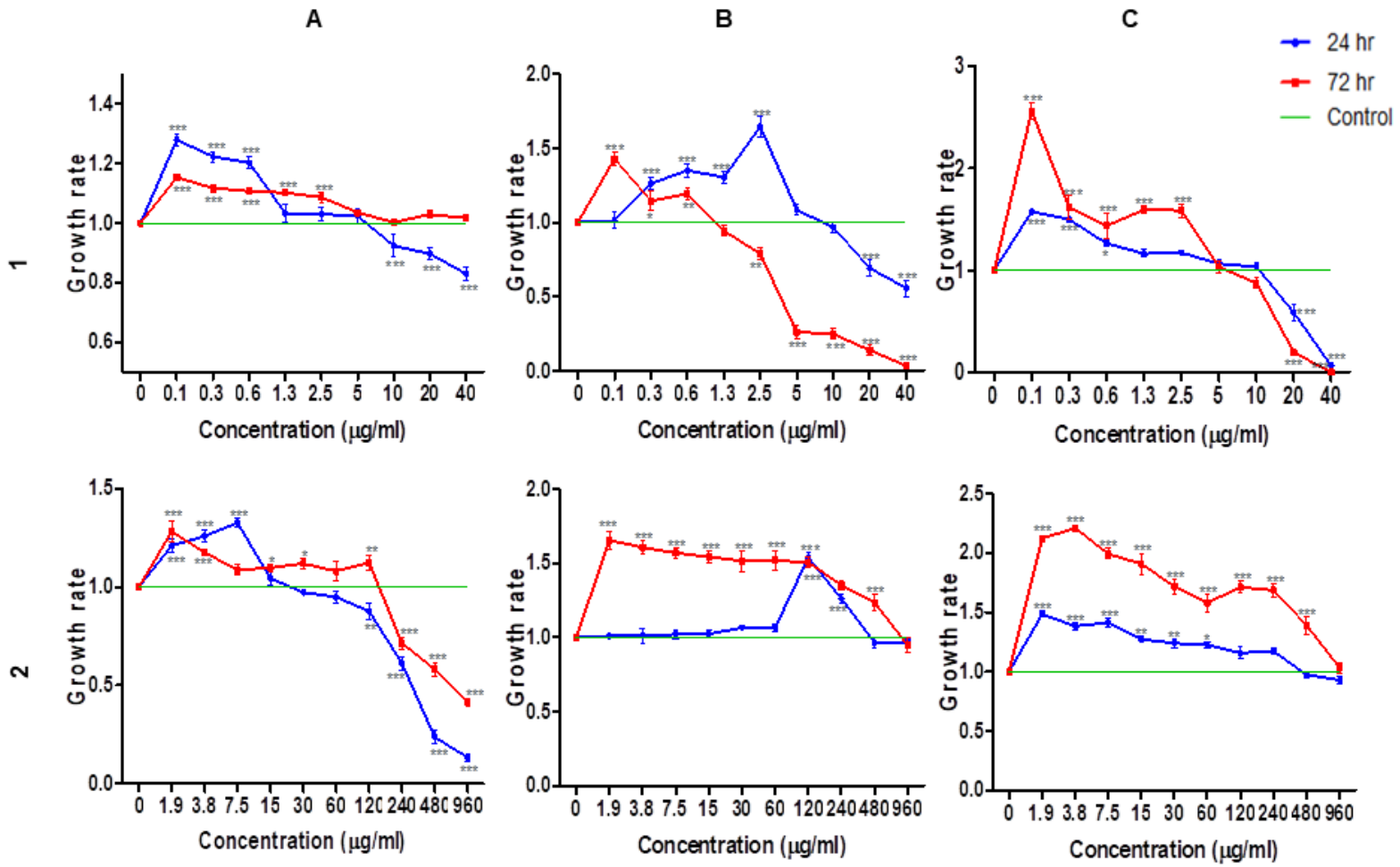


Figure 1. Effects of (**1**) *Cotyledon*-AgNPs and (**2**) *C. orbiculata* water extract on the cell viability and growth rate of (**A**) KMST-6, (**B**) HaCaT and (**C**) CHO cells after 24 and 72 h. Each value represents mean ± standard error of the mean (SEM); statistical significance of the *C. orbiculata* water extract and *Cotyledon*-AgNPs-treated cells when compared to the untreated cells is indicated with * for $p < 0.05$, ** p for <0.01, and *** $p < 0.001$.

2.3. Wound Healing Activity

The wound healing activity of the *C. orbiculata* water extracts and *Cotyledon*-AgNPs was tested using the scratch assay. This is a popular, simple method used to determine the wound-healing activities of different compounds [45]. It is related to the proliferation phase of wound healing which involves the migration and proliferation of fibroblasts and keratinocytes. This assay mimics the in vivo migration of cells during wound healing [46]. Both the *C. orbiculata* water extracts and the *Cotyledon*-AgNPs were able to close the scratched gap faster than the Negative Control (untreated cells) in all cell lines (Figure 2). The images show a time-dependent increase in the density of cells in the scratched area until the gap closes. This strongly indicates that the *C. orbiculata* water extracts and the *Cotyledon*-AgNPs possess both cell migration and proliferation properties. Therefore, it can be concluded that *C. orbiculata* promotes wound healing activities via increased migration and proliferation of fibroblasts, keratinocytes and epithelial cells. Our findings support literature that reports that AgNPs promote wound healing through the differentiation of myofibroblasts to fibroblasts, wound contractility and epidermal re-epithelialization through keratinocyte proliferation and migration [2,47]. Similar observations where plant-derived AgNPs promoted wound healing via migration of fibroblasts [48,49] and keratinocytes [50] have been previously reported. In HaCaT and CHO cells, *Cotyledon*-AgNPs (at 2.5 µg/mL) exhibited more activity than the water extract, in-fact at this concentration, *Cotyledon*-AgNPs had more activity than the Positive Control (treatment with 15 µg/mL allantoin) (Figures 2 and 3). Chinnasamy et al. 2019 reported similar results with *Melia azedarach* synthesized AgNPs which exhibited increased wound healing activities compared to the *M. azedarach* extract alone [51]. This might be due to the nanoparticles' small size which increases their surface area to volume ratio thus increasing their activity [2]. It might also be due to phytochemicals present on the nanoparticles (such as polyphenols and flavonols as shown in Table 1) which can increase their bioavailability and enhance their activity [52]. In KMST-6 cells, however, the water extract showed more activity than the *Cotyledon*-AgNPs. However, considering that a lower concentration of the AgNPs (2.5 µg/mL) was used; the latter statement cannot be confirmed. Although emphCotyledon-AgNPs were more active than the *C. orbiculata* water extract, the activity of the extract was also significant as it showed good activity at very low concentrations ($\leq$15 µg/mL). These findings show that *C. orbiculata* most likely contains compounds with highly active wound-healing properties, and therefore, provide some scientific evidence supporting its traditional use. Other medicinal plants were also recorded to have good wound healing activity, these include *Plantago australis* [53], *Spirulina platensis* [54], *Alternanthera sessilis* [55], *Commiphora molmol*, *Aloe vera* [56] and *Achillea eriophora* [57].

Figures 2 and 3 show that all cell lines exhibited different migration rates. It can be noted that complete gap closure of HaCaT, CHO and KMST-6 cells took 48, 72 and 96 h, respectively (Figure 3). The daily percentage wound closure rates were determined using the equation stated in Section 3.5.3, these rates correspond to the findings from the scratch assay (Figure 2). After exposure to the *C. orbiculata* water extract and the *Cotyledon*-AgNPs, HaCaT and CHO cells responded in a similar pattern, while the response of KMST-6 cells was different. In these cells, the wound closure rates were increased in the first 24 h compared to KMST-6 cells which had a more evenly spread cell migration pattern. The results in Figure 3 where almost complete wound closure was observed in HaCaT cells after day 1 corresponds to the increased growth rate in *Cotyledon*-AgNPs (2.5 µg/mL) treated HaCaT cells after 24 h as shown in Figure 1.

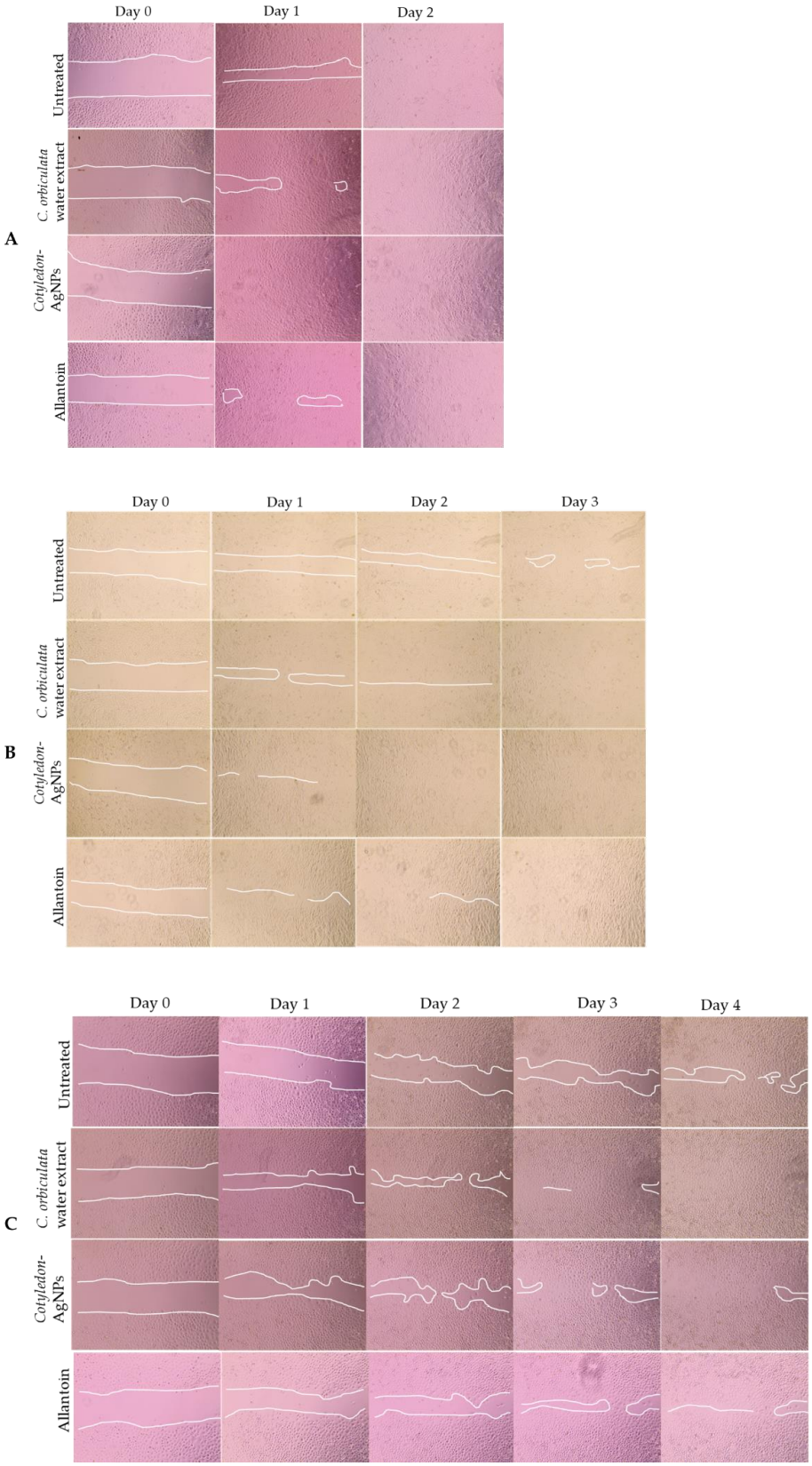


Figure 2. Scratch assay analysis of *Cotyledon*-AgNPs (2.5 μg/mL) and *C. orbiculata* water extract (15 μg/mL) on HaCaT (**A**), CHO (**B**) and KMST-6 (**C**) cells. The white lines in the images are boundaries of the scratched gap.

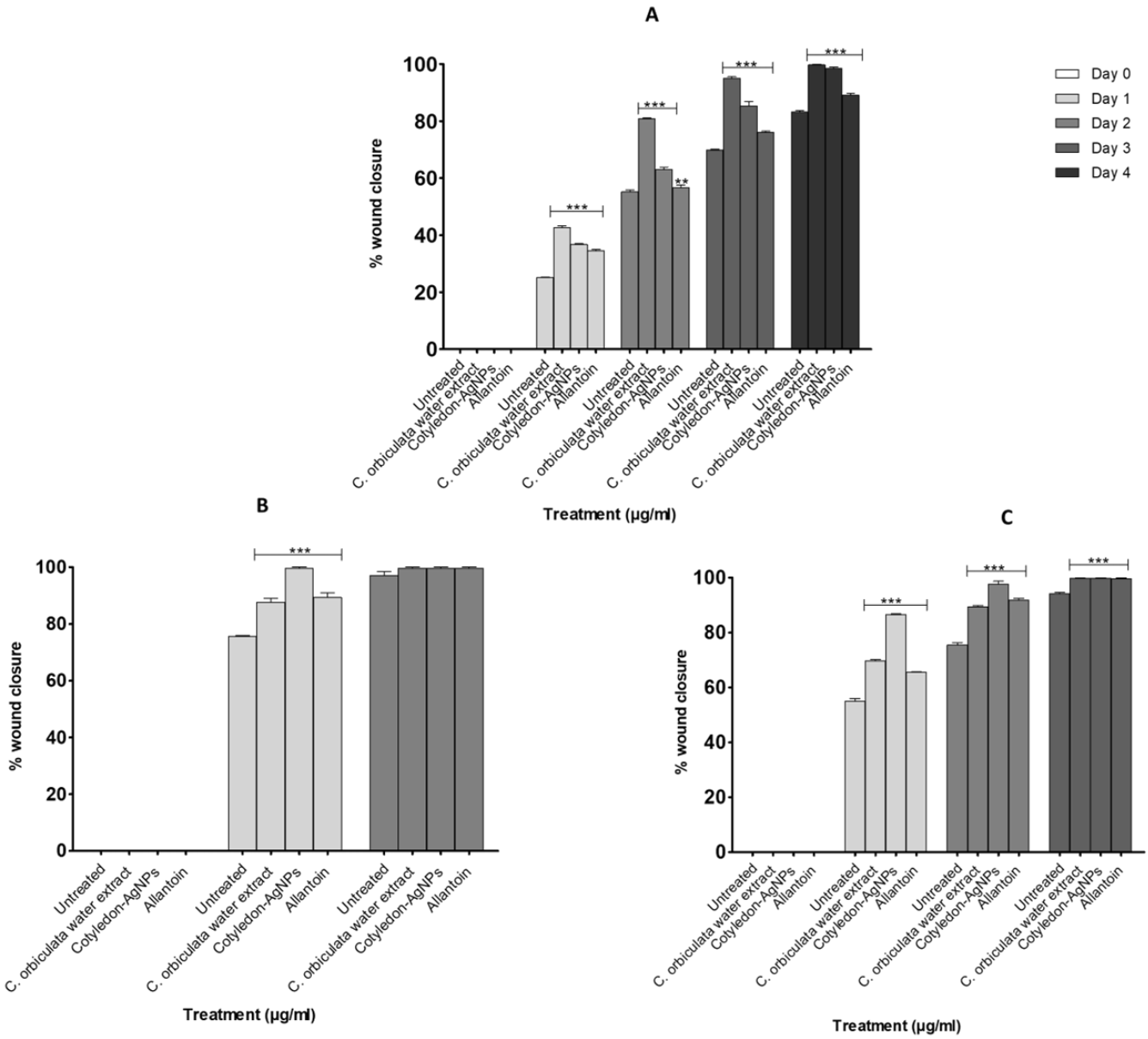


Figure 3. Percentage wound closure rates of *Cotyledon*-AgNPs and *C. orbiculata* water extract-treated KMST-6 (**A**), HaCaT (**B**) and CHO (**C**) cells. Each value represents mean ± standard error of the mean (SEM); statistical significance was indicated with ** p for < 0.01 and *** $p < 0.001$.

2.4. Gene Expression Studies

KMST-6 (fibroblasts) cells were used for gene expression studies. This is because fibroblasts are critical in all stages of wound healing and are involved in key processes such as extracellular matrix production, collagen deposition and wound contraction [58,59]. Gene expression studies investigating the expression levels of 86 wound healing-related genes were conducted on KMST-6 cells. In *Cotyledon*-AgNPs treated cells, only 17 of the 86 genes were differentially expressed (Figure 4). Eight (CDH1, COL14A1, EGF, FGA, FGF10, ITGB1, ITGB6, PTGS2) of the differentially expressed genes (DEGs) were upregulated while the other nine (CCL2, CTGF, CXCL2, FGF2, HBEGF, IL6, ITGA2, MMP2, SERPINE1) were downregulated (Figures 4 and 5). Genes with a fold change of $\geq \pm 1.5$ and p-values of <0.05 were considered DEGs. DAVID and STRING analysis clustered DEGs into three different groups (Figure 6). Cluster A consisted of five downregulated genes (CCL2, CXCL2, IL6, MMP2, SERPINE1) and one upregulated gene (PTGS2). These five genes are involved in

the inflammatory phase of wound healing. Cluster A genes promote inflammation, mostly via leucocyte chemotaxis, cytokine production and prostaglandin production [60–62]. Although inflammation plays an important role during the initial stages of wound healing, it is known that extended inflammation can lead to chronic wounds [63]. Based on the gene expression study which shows that most of the genes involved in inflammation are down-regulated it can be concluded that the *Cotyledon*-AgNPs have anti-inflammatory activity. This is in agreement with our previous study which demonstrated the anti-inflammatory activity of *Cotyledon*-AgNPs in THP-1 macrophages [25].

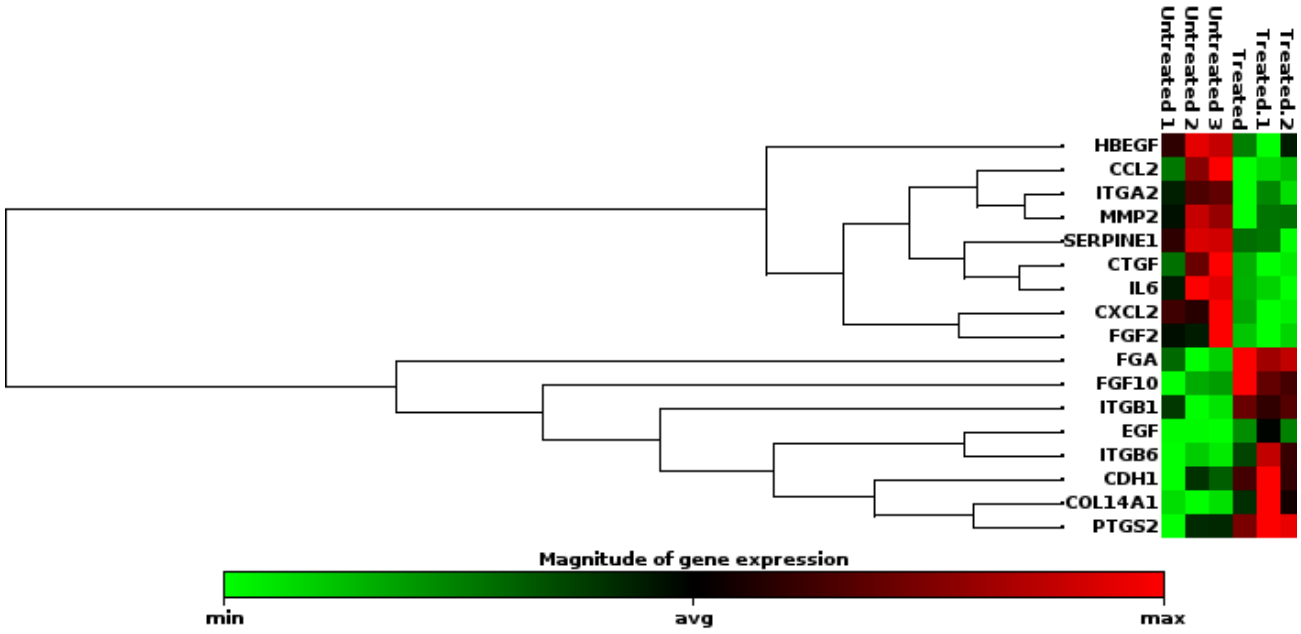


Figure 4. Clustergram of DEGs expressed in *Cotyledon*-AgNPs treated KMST-6 cells.

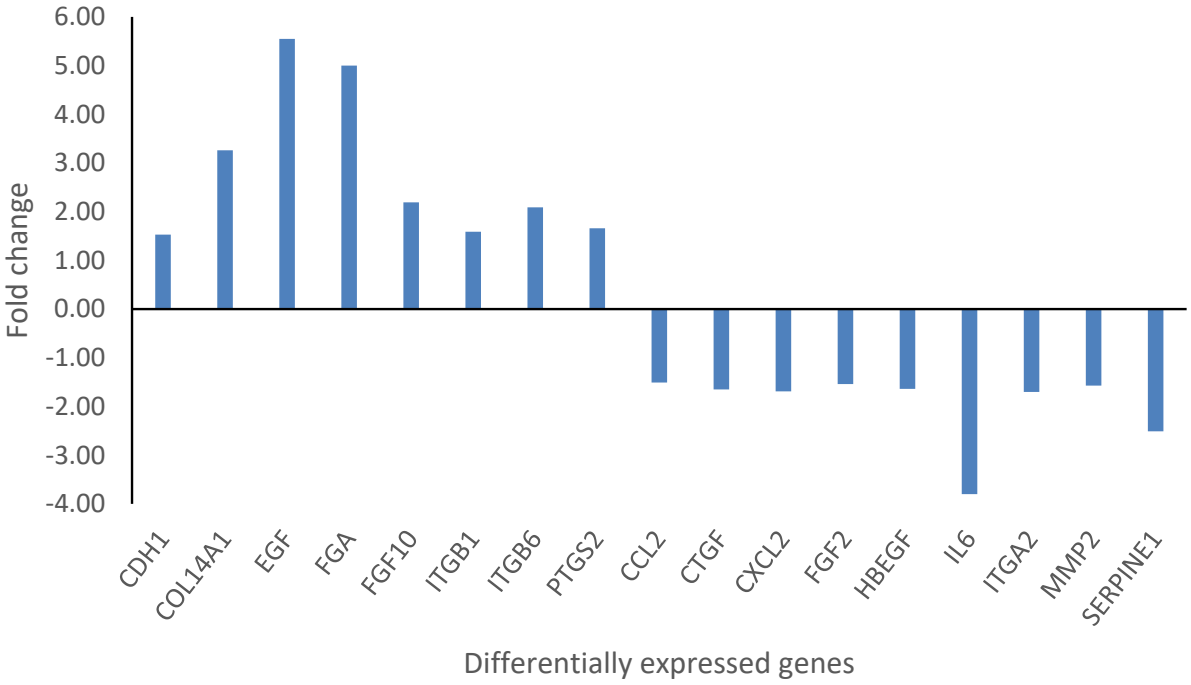


Figure 5. Fold changes in the DEGs expressed after KMST-6 cells were treated with *Cotyledon*-AgNPs. Genes with a fold change of $\geq \pm 1.5$ and p-values of <0.05 were considered DEGs.

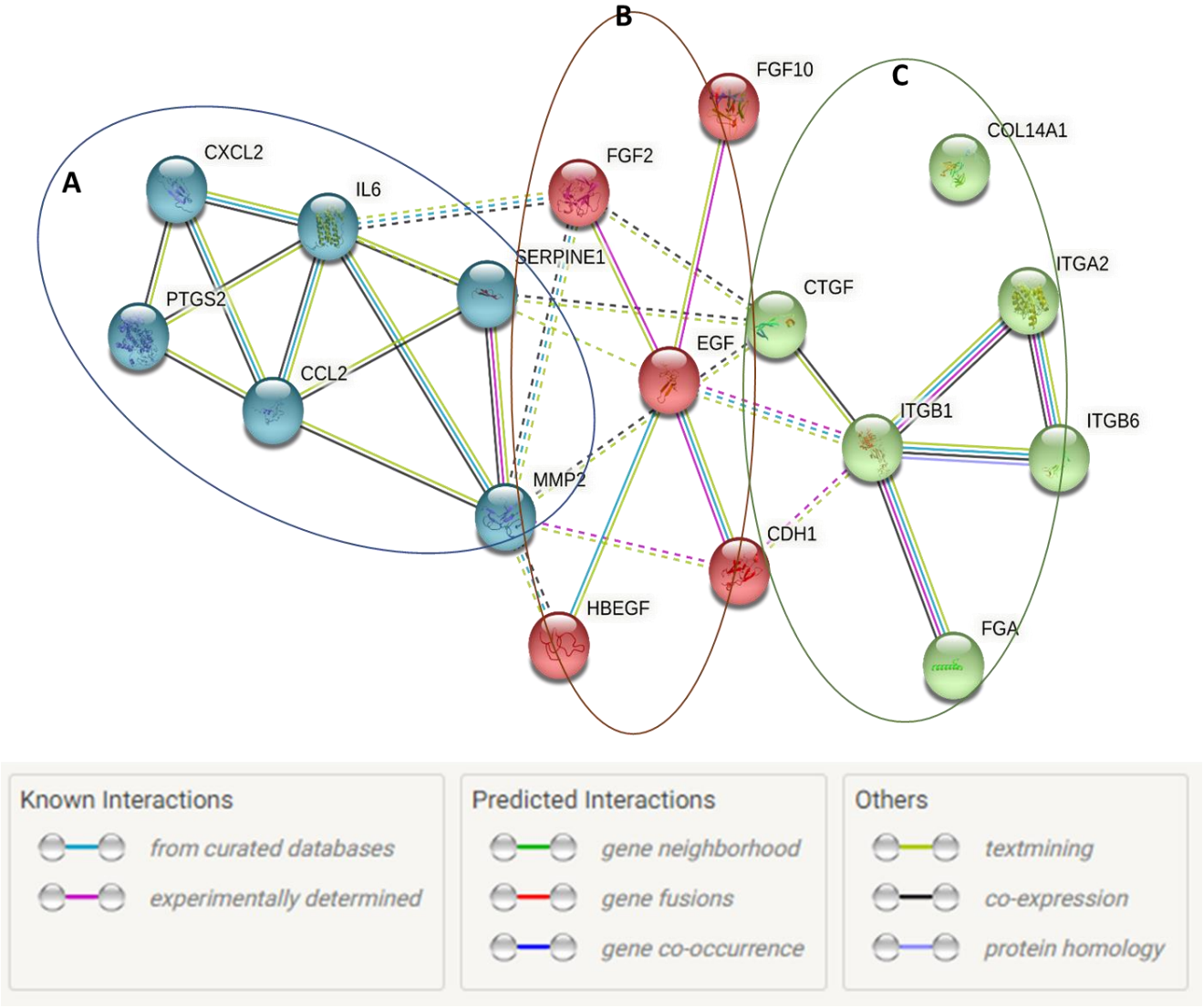


Figure 6. Protein networks showing the interactions between DEGs in the *Cotyledon*-AgNPs treated KMST-6 cells. These networks were determined using the STRING database.

As shown in Table 2, the downregulated genes function in a number of pathways, including TNF signaling and cytokine production, all of which lead to inflammation.

Table 2. Enriched pathways in which the *Cotyledon*-AgNPs expressed DEGs function through.

Pathways	Involved Genes	*p*-Value
PI3K-Akt signalling pathway	EGF, FGF10, FGF2, ITGB1, ITGB6, ITGA2, IL6	7.9×10^{-5}
Regulation of actin cytoskeleton	EGF, FGF10, FGF2, ITGB1, ITGB6, ITGA2	8.4×10^{-5}
Rap1 signalling pathway	EGF, FGF10, FGF2, ITGB1, CHD1	1.2×10^{-3}
Focal adhesion	ITGA2, ITGB1, ITGB6, EGF	1.1×10^{-2}
ECM-receptor interaction	ITGA2, ITGB1, ITGB6	1.7×10^{-2}
IL-17 signalling pathway	CXCL2, CCL2, IL6, PTGS2	4.5×10^{-5}
TNF signaling pathway	CXCL2, CCL2, IL6, PTGS2	1.8×10^{-3}
NOD-like receptor signaling pathway	CXCL2, CCL2, IL6	7.3×10^{-3}
Cytokine-cytokine receptor interaction	CXCL2, CCL2, IL6	1.6×10^{-2}

Cluster B contained three (CDH1, EGF, FGF10) upregulated and two (FGF-2, HB-EGF) downregulated genes. Genes in this cluster are mainly involved in epithelial cell proliferation and migration. The upregulated genes stimulate keratinocyte proliferation and migra-

tion and also increase the tensile strength of the new skin [64–66]. These genes also improve collagen construction and stimulate the formation of the extracellular matrix (ECM) [64,67]. It is possible that the downregulated genes are downstream of the upregulated ones and may, therefore, be activated with increased exposure to the *Cotyledon*-AgNPs. Although the downregulated genes promote keratinocyte proliferation and migration, they also promote inflammation [68,69]. This might be the reason why they were downregulated. These genes modulate inflammatory cell recruitment and activation, particularly macrophages and fibroblasts [69,70]. Upon activation, fibroblasts produce and secrete pro-inflammatory cytokines (IL6, IL-1B, IL8), chemokines (CCL2) and prostanoids (prostaglandin E2) [71–73].

Cluster B genes highly correlate with those in cluster C, these genes function using the same pathways (PI3K-Akt signaling pathway, Regulation of actin cytoskeleton, Rap1 signaling pathway and Focal adhesion) as shown in Table 2. Group C genes are involved in cellular adhesion which regulates cell differentiation and migration [74]. Cell adhesion is the ability of cells to stick to each other or to the ECM through molecules such as collagen, fibronectin, and laminin [75]. It is essential in cell communication and regulation as well as in the development and maintenance of tissues. Cell adhesion is also essential in wound healing as it stimulates signals that regulate important wound-healing processes such as cell differentiation, migration and survival [74]. Integrins are the main mediators of cell attachment to the ECM. They form cellular receptors for the extracellular environment and adjacent cells [76]. Their binding to extracellular ligands such as fibronectin and laminin promotes intracellular signaling which eventually causes cell migration [77]. The upregulated integrin genes ITGB1, ITGB6 and FGA are not only essential in cell adhesion but also in angiogenesis. FGA leads to the formation of an insoluble fibrin matrix. The major function of fibrin is its involvement in the formation of blood clots, therefore, reducing blood loss. Fibrin also forms the temporary ECM in the wounded area and plays important roles in tissue repair and cell migration during angiogenesis [78].

The effects of the *C. orbiculata* water extract on wound healing genes were also determined. Of the 86 genes tested, only 11 (COL5A3, ACTC1, FGF7, WNT5A, ITGB6, TAGLN, ITGB1, VTN, ITGA4, CSF2, FGF10) were differentially expressed; all of them were upregulated (Figures 7 and 8). Two (FGF10, ITGB1) of these genes were also upregulated in *Cotyledon*-AgNPs treated cells (Figure 5), both genes are involved in the proliferation and migration of epithelial cells. Both STRING and the DAVID software were used to analyze these results. These upregulated genes are separated into three clusters all of which promote cell proliferation and migration (Figure 9). Cluster A genes (particularly FGF10 and WNT5A) are involved in epithelial cell proliferation and migration especially fibroblasts and keratinocytes. The WNT protein family is made up of glycoproteins that regulate cell proliferation, in this family WNT5A was discovered as a key regulator of fibroblast proliferation [79,80].

Cluster B genes are involved in cell adhesion mostly by integrin, leading to increased cell survival. Cluster C consists of two genes (ACTA1 and TAGLN) which are involved in cytoskeleton organization and cell motility [81]. Actin is a structural protein that makes up the cell cytoskeleton while TAGLN is an actin crosslinking protein [81,82]. These genes ultimately support cell differentiation, proliferation, and migration [83]. Genes differentially expressed in response to treatment with *C. orbiculata* water extract were in similar categories to those expressed by the *Cotyledon*-AgNPs (cluster B, FGF10 and cluster C, ITGB1), they function using similar pathways as shown in Tables 2 and 3. This shows that the *C. orbiculata* water extract and the *Cotyledon*-AgNPs promote wound healing using similar mechanisms which include cell proliferation and migration. This can confirm the results in Table 1 which show that the same phytochemicals present in the extract and responsible for the bioactivities are also present on the nanoparticles. Results obtained using the WST-1 assay (Figure 1) and the scratch assay (Figure 2) support this finding.

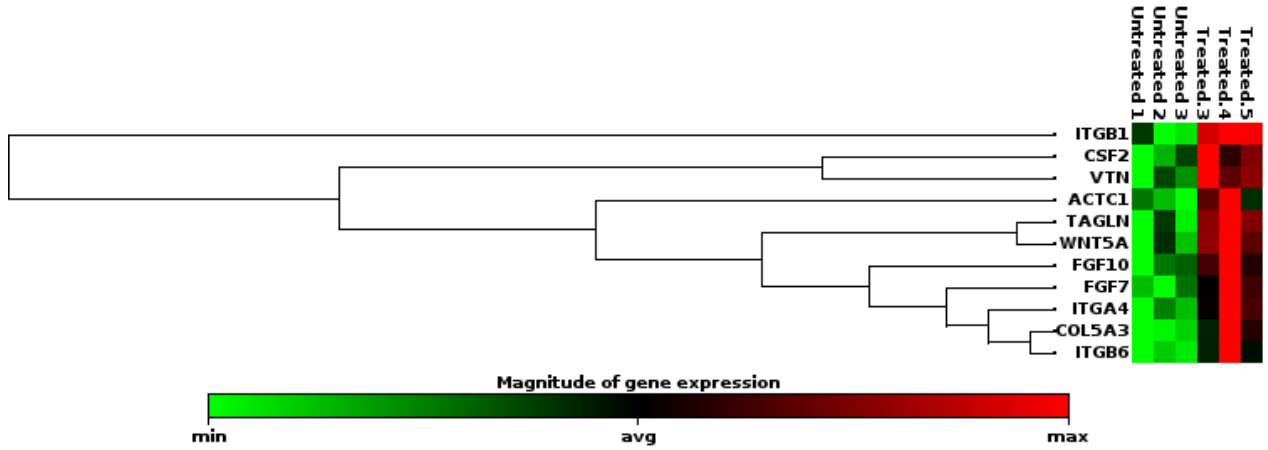


Figure 7. Clustergram of DEGs expressed in *C. orbiculata* water extract-treated KMST-6 cells.

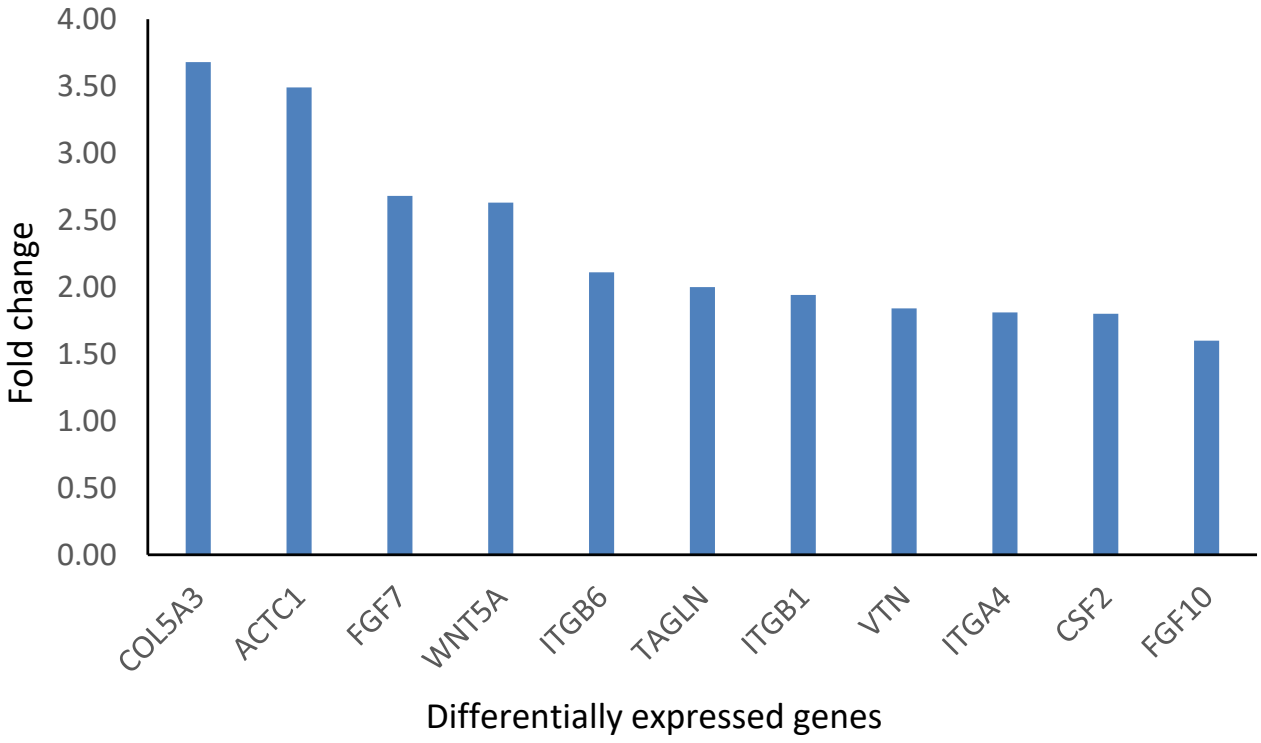


Figure 8. Fold changes in the DEGs expressed after KMST-6 cells were treated with *C. orbiculata* water extract. Genes with a fold change of $\geq \pm 1.5$ and p-values of <0.05 were considered as DEGs.

Table 3. Enriched pathways in which *C. orbiculata* water extract expressed DEGs function through.

Pathways	Involved Genes	*p*-Value
PI3K-Akt signalling pathway	FGF10, FGF7, COL5A3, ITGB1, ITGB6, ITGA4, VTN	1.1×10^{-6}
Regulation of actin cytoskeleton	FGF10, FGF7, ITGB1, ITGB6, ITGA4	9.4×10^{-5}
ECM-receptor interaction	ITGB1, ITGB6, ITGA4, COL5A3, VTN	2.9×10^{-6}
Focal adhesion	ITGB1, ITGB6, ITGA4, COL5A3, VTN	8.7×10^{-5}
Rap1 signalling pathway	FGF10, FGF7, ITGB1	2.9×10^{-2}

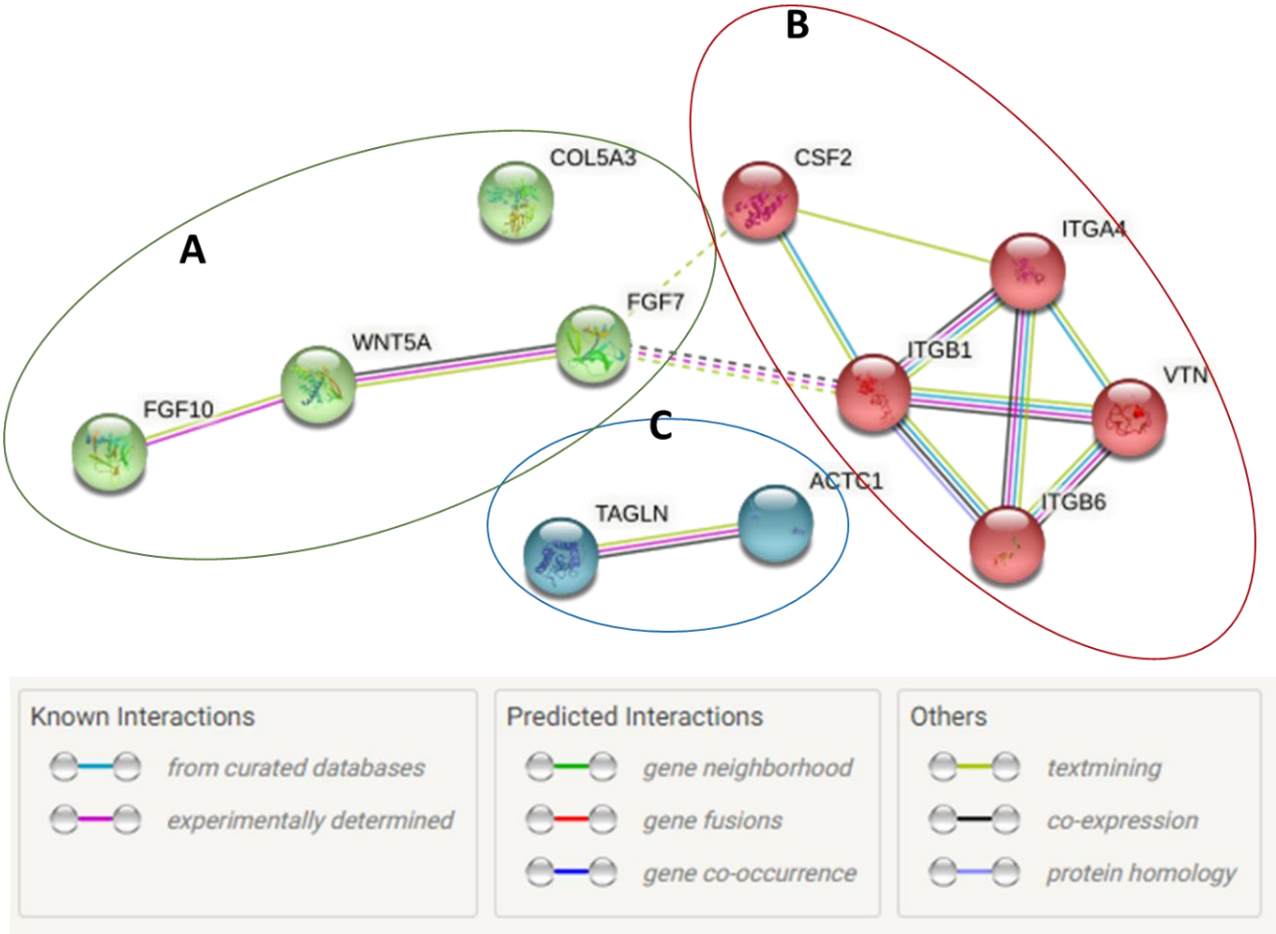


Figure 9. Protein networks showing the interactions between DEGs expressed in KMST-6 cells treated with *C. orbiculata* water extract. These networks were determined using the STRING database.

3. Materials and Methods

3.1. Materials

The Folin–Ciocalteu reagent, Sodium carbonate, 4-Dimethylamino-cinnamaldehyde, Methanol, Hydrochloric acid, Acetate buffer, 2, 4, 6-tripyridyl-S-triazine, Ascorbic acid, ABTS radical, WST-1 reagent, Hams-F12 medium, Dulbecco's modified eagle medium (DMEM), Phosphate-buffered saline (PBS), Fetal bovine serum (FBS), Pen-strep (penicillin and streptomycin), were obtained from Sigma-Aldrich (St. Louis, Mo, USA). Gene expressions kits (RNeasy Mini Kit, RT2 First Strand Kit, Human wound healing RT2 Profiler PCR Array) were from Qiagen (Hilden, Germany).

3.2. Plant Extract Preparation and Synthesis of Cotyledon-AgNPs

The plant extracts and *Cotyledon*-AgNPs used in this study were prepared as described previously [25].

3.3. Phytochemical Analysis

3.3.1. TPC

The TPC of the *C. orbiculata* water extract and the *Cotyledon*-AgNPs was determined using the Folin–Ciocalteu method [84]. In a 96-well plate, 25 µL of the samples and standards were mixed with 125 µL of the Folin–Ciocalteu reagent (10%). After 5 min, 100 µL of sodium carbonate was added to the mixture. The plates were incubated for 2 h at room temperature and then read using a spectrophotometer at an absorbance of 765 nm.

3.3.2. Total Flavonoid Content (TFC)

The Flavanol Content

The flavanol content was determined using the method by [85] with modifications. In short, 50 µL of the *C. orbiculata* extract and *Cotyledon*-AgNPs were mixed with 250 µL of 4-Dimethylamino-cinnamaldehyde. The mixture was incubated for 30 min at room temperature after which the absorbance was read at 640 nm. Results were expressed as milligram catechin equivalent per gram (mg CE/g).

The Flavonol Content

The flavonol content was also determined according to the method in [85], with modifications. Briefly, 12.5 µL of 0.1% HCl in methanol and 225 µL of 2% HCl solutions were added to 12.5 µL of the *C. orbiculata* extract and *Cotyledon*-AgNPs. The mixture was incubated for 30 min at room temperature and the absorbance was read at 360 nm. Results were expressed as milligram quercetin equivalent per gram (mg QE/g).

3.4. Antioxidant Studies

The antioxidant activity of the *C. orbiculata* water extract and the *Cotyledon*-AgNPs was determined using the most common colorimetric assays namely FRAP and ABTS.

3.4.1. FRAP

This method is based on the ability of antioxidants to reduce the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). In the presence of antioxidants at low pH, the ferric tripyridyl triazine complex (colorless) is reduced to its ferrous form which is a blue-colored complex. The ferric-reducing power of the *C. orbiculata* water extract and *Cotyledon*-AgNPs was evaluated according to the method developed by Benzi and Strain in 1996 [86]. The FRAP reagent was prepared by mixing 10 mL of acetate buffer (300 mM), 1 mL of 2, 4, 6-tripyridyl-S-triazine (TPTZ) (10 mM) and 1 mL of $FeCl_3 \cdot 6H_2O$ (20mM). In a 96-well plate, the FRAP solution (300 µL) was mixed with 10 µL of the samples (*C. orbiculata* water extract and *Cotyledon*-AgNPs) and incubated at 37 °C for 30 min. A spectrophotometer was used to measure the absorbance readings at 593 nm. Ascorbic acid was used in the preparation of the standard solutions. The absorbance of the samples was compared to a standard curve and the values were expressed as µmol AAE/g.

3.4.2. ABTS

The ABTS assay is used to measure the total antioxidant activity of substances. It is based on the ability of antioxidants to scavenge the ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation relative to the Trolox standard. This assay was performed according to the method used by Dube et al. 2017 [84]. Briefly, the ABTS radical mixture was prepared by mixing 5 mL ABTS (7 mM) and 88 µL potassium peroxodisulfate ($K_2S_2O_8$) (140 mM). The mixture was incubated in the dark for 24 h at room temperature and was diluted with ethanol to read an absorbance of approximately 2.0 ($\pm$0.1) at 734 nm, as the control; 300 µL of the diluted ABTS radical mixture was added to 25 µL of the *C. orbiculata* extracts, *Cotyledon*-AgNPs and standard in 96 well plates. The plates were incubated for 30 min at room temperature and absorbance readings were taken at 734 nm. The results were expressed as µmol TE/g.

3.5. Cell Culture Studies

3.5.1. Cell Culture

HaCaT, KMST-6 and CHO cells were obtained from the DSI/Mintek NIC (Nanotechnology Innovation Centre) laboratory at the University of the Western Cape (South Africa). The HaCaT and KMST-6 cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% Fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. The CHO cells were grown in a Hams-F12 medium containing 10% FBS and 1% Penicillin/Streptomycin. All the cells were maintained in a humidi-

fied atmosphere of 5% CO_2 in a 37 °C incubator (SL SHEL LAB, Sheldon manufacturing, Cornelius, OR, USA).

3.5.2. Cell Viability and Growth Rate

Both cell viability and growth rate were determined using the WST-1 (Sigma-Aldrich, St. Louis, MO, USA) assay according to the method by [87]. Cells were seeded in 96 well plates at a density of 1×10^5 cell/mL and incubated for 24 h. The growth media was replaced with a medium containing *C. orbiculata* water extract and *Cotyledon*-AgNPs at different concentrations and the cells were further incubated for 24 to 72 h. After incubation, the conditioned media was replaced with 10% WST-1 reagent (diluted in respective medium) and then plates were incubated for an additional 3 h. The absorbance was measured at 440 nm (reference 630 nm) using a microplate reader (POLARstar Omega plate reader, BMG-Labtech, Ortenberg, Germany). Cell viability was expressed as a percentage of the absorbance of treated cells to control (untreated) cells. The cell growth rate was determined using cell viability; 100% cell viability (untreated cells) was used as the control for growth rate with a value of 1.

3.5.3. Scratch Assay

The scratch assay was determined using a method by [55], with modifications. HaCaT, KMST-6 and CHO cells were seeded in 24 well plates at a density of 2×10^5 cell/mL and were incubated for 24 h at 37 °C in 5% CO_2. After 24 h, a monolayer of confluent cells was observed. To create a scratch, the cell monolayer was scraped in a straight line using a sterile pipette tip. Cellular debris was removed by washing the wells with fresh medium. The cells were incubated at 37 °C in the presence of *C. orbiculata* water extract and the *Cotyledon*-AgNPs. The concentrations of *C. orbiculata* water extract and the *Cotyledon*-AgNPs were 15 and 2.5 µg/mL, respectively. Allantoin (15 µg/mL) which is a plant-derived commercial drug and known to induce cell growth was used as a positive control [55]. Untreated scratched cells were used as the negative control. All treatments and controls were placed in a medium containing 1% FBS. The assay was conducted in triplicate. Wound closure was monitored at different time intervals using a digital light microscope (EVOS XL Core Cell Imaging System, CA, USA). Images were analyzed using the Image J software. The percentage of wound closure was obtained using the equation,

$$\text{Wound closure } (\%) = \frac{\text{Wound area } (0\text{ h}) - \text{ Wound area } (\text{t h})}{\text{Wound area } (0\text{ h})} \times 100$$

3.6. Gene Expression Studies

KMST-6 cells were seeded at a density of 2×10^5 cell/mL in a 25 cm^2 cell culture flask, the flasks were treated with *C. orbiculata* water extracts (15 µg/mL) and *Cotyledon*-AgNPs (2.5 µg/mL). Untreated flasks were used as the control. This was conducted in triplicate. The cells were collected into Eppendorf tubes and prepared for RNA extraction. Total RNA was extracted from the untreated and treated cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration of the RNA was determined using a Qubit® 2.0 Fluorometer (Invitrogen by Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized from RNA using the RT2 First Strand Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The produced cDNA was used as a template for RT-qPCR which was conducted using the Human wound healing RT2 Profiler PCR Array (Qiagen, Hilden, Germany) following the manufacturer's instructions. The experiment was carried out on the Roche LightCycler 480 (Roche, Basel, Switzerland) in the department of the Institute of Microbial Biotechnology and Metagenomics (IMBM) at the University of the Western Cape. Data collected from the LightCycler 480 was analyzed at GeneGlobe Data Analysis Center under Qiagen.

The relative changes in gene expression were calculated using cycle threshold (CT) values. The CT values were first normalized to those of used housekeeping genes. The fold change was then calculated using the $2^{-\Delta\Delta CT}$formula adapted from Livak and Schmittgen [88]. Genes with a fold change of $\geq \pm 1.5$ and p-values of <0.05 were considered as DEGs and were used for further analysis. The Database for Annotation, Visualization and Integrated Discovery (DAVID; version 6.7) and Search Tool for the Retrieval of Interaction Genes/Proteins (STRING; https://string-db.org/, accessed on 5 November 2022) pathways were used to further analyze the different interactions of the DEGs.

3.7. Statistical Analysis

Statistical analysis of the data was conducted using the GraphPad Prism 6 software. All assays were performed in triplicates and the results were expressed as mean ± standard error of the mean (SEM). Statistical significance was indicated as * for $p < 0.05$, ** p for < 0.01, *** $p < 0.001$ and **** for $p < 0.0001$.

4. Conclusions

It can be concluded that the medicinal plant, *C. orbiculata* possesses antioxidant and wound-healing activities. Both the *C. orbiculata* water extract and the *Cotyledon*-AgNPs showed good antioxidant activities which can increase their wound healing capabilities. They both increased the growth of HaCaT, KMST-6 and CHO cells at low concentrations and promoted cell migration in these cells in the scratch assay. The activity of the *Cotyledon*-AgNPs can be attributed to their small size which increases their surface area to volume ratio, hence increasing their activity. It might also be due to phytochemicals present on the nanoparticles which can increase their bioavailability and enhance their activity. Gene expression studies confirmed that the *C. orbiculata* water extract and *Cotyledon*-AgNPs promote wound healing through the activation of genes involved in the proliferation phase of wound healing. *C. orbiculata* extract and *Cotyledon*-AgNPs promote keratinocyte and fibroblast proliferation and migration by upregulating genes such as FGF7 and FGF10. *Cotyledon*-AgNPs are also involved in hemostasis which promotes clotting thus reducing blood loss. While *C. orbiculata* has been used in traditional medicine to treat skin conditions and wounds, this study scientifically shows that this plant contains wound-healing properties. We previously demonstrated that *Cotyledon*-AgNPs have significant antibacterial activity against microorganisms that commonly infect wounds. We also showed previously that these nanoparticles have anti-inflammatory activity. The current study shows that the *Cotyledon*-AgNPs also promote the growth of keratinocytes and fibroblasts at specific concentrations. We have also shown that these nanoparticles are not toxic at these concentrations. Taken together these findings demonstrate that *Cotyledon*-AgNPs have immense potential for application as novel wound healing agents.

Author Contributions: Conceptualization, M.M. and S.M.; investigation, C.T.; resources, M.M. and S.M.; writing—original draft preparation, C.T.; writing—review and editing, C.T., A.D.W., A.M.M., M.M. and S.M.; supervision, M.M. and S.M.; funding acquisition, M.M. and S.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the South African National Research Foundation (grant number 114598 and 106642) and the DSI/MINTEK Nanotechnology Innovation Centre (NIC).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Supporting data presented in this study are available on request from the corresponding author.

Acknowledgments: We thank the Institute for Microbial Biotechnology and Metagenomics (UWC) and Bronwyn Kirby for granting us access to the Roche LightCycler 480 instrument. The authors also wish to thank Fanie Rautenbach (CPUT) for his assistance with the antioxidant studies.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Verma, A.; Gautam, S.; Bansal, K.; Prabhakar, N.; Rosenholm, J. Green Nanotechnology: Advancement in Phytoformulation Research. *Medicines* **2019**, *6*, 39. [CrossRef] [PubMed]
2. Mihai, M.M.; Dima, M.B.; Dima, B.; Holban, A.M. Nanomaterials for Wound Healing and Infection Control. *Materials* **2019**, *12*, 2176. [CrossRef] [PubMed]
3. Siddiqi, K.S.; ur Rahman, A.; Tajuddin; Husen, A. Properties of Zinc Oxide Nanoparticles and Their Activity Against Microbes. *Nanoscale Res. Lett.* **2018**, *13*, 141. [CrossRef]
4. Konop, M.; Damps, T.; Misicka, A.; Rudnicka, L. Certain Aspects of Silver and Silver Nanoparticles in Wound Care: A Minireview. *J. Nanomater.* **2016**, *2016*, 1–10. [CrossRef]
5. Gunasekaran, T.; Nigusse, T.; Dhanaraju, M.D. Silver Nanoparticles as Real Topical Bullets for Wound Healing. *J. Am. Coll. Clin. Wound Spec.* **2012**, *3*, 82–96. [CrossRef]
6. Street, R.A.; Prinsloo, G. Commercially Important Medicinal Plants of South Africa: A Review. *J. Chem.* **2013**, *2013*, 1–16. [CrossRef]
7. Louw, C.A.M.; Regnier, T.J.C.; Korsten, L. Medicinal Bulbous Plants of South Africa and Their Traditional Relevance in the Control of Infectious Diseases. *J. Ethnopharmacol.* **2002**, *82*, 147–154. [CrossRef]
8. Maroyi, A. A Review of Botany, Medicinal Uses, Phytochemistry and Biological Activities of Cotyledon Orbiculata. *J. Pharm. Sci. Res.* **2019**, *11*, 3491–3496.
9. Thring, T.S.A.; Weitz, F.M. Medicinal Plant Use in the Bredasdorp/Elim Region of the Southern Overberg in the Western Cape Province of South Africa. *J. Ethnopharmacol.* **2006**, *103*, 261–275. [CrossRef]
10. Kumari, A.; Baskaran, P.; Van Staden, J. In Vitro Propagation and Antibacterial Activity in Cotyledon Orbiculata: A Valuable Medicinal Plant. *Plant Cell Tissue Organ Cult.* **2016**, *124*, 97–104. [CrossRef]
11. Ondua, M.; Njoya, E.M.; Abdalla, M.A.; McGaw, L.J. Anti-Inflammatory and Antioxidant Properties of Leaf Extracts of Eleven South African Medicinal Plants Used Traditionally to Treat Inflammation. *J. Ethnopharmacol.* **2019**, *234*, 27–35. [CrossRef] [PubMed]
12. Molefe, N.I.; Tsotetsi, A.M.; Ashafa, A.O.T.; Thekisoe, O.M.M. In Vitro Anthelmintic Activity of Cotyledon Orbiculata, Hermannia Depressa and Nicotiana Glauca Extracts against Parasitic Gastrointestinal Nematodes of Livestock. *J. Med. Plants Res.* **2013**, *7*, 536–542. [CrossRef]
13. Mhlongo, F.; Cordero-maldonado, M.L.; Crawford, A.D.; Katerere, D.; Sandasi, M.; Hattingh, A.C.; Koekemoer, T.C.; Venter, M.V.D.; Viljoen, A.M. Evaluation of the Wound Healing Properties of South African Medicinal Plants Using Zebrafish and in Vitro Bioassays. *J. Ethnopharmacol.* **2022**, *286*, 114867. [CrossRef] [PubMed]
14. Desmet, C.M.; Préat, V.; Gallez, B. Nanomedicines and Gene Therapy for the Delivery of Growth Factors to Improve Perfusion and Oxygenation in Wound Healing. *Adv. Drug Deliv. Rev.* **2018**, *129*, 262–284. [CrossRef] [PubMed]
15. Shedoeva, A.; Leavesley, D.; Upton, Z.; Fan, C. Wound Healing and the Use of Medicinal Plants. *Evid.-Based Complementary Altern. Med.* **2019**, *2019*, 2684108. [CrossRef] [PubMed]
16. Han, G.; Ceilley, R. Chronic Wound Healing: A Review of Current Management and Treatments. *Adv. Ther.* **2017**, *34*, 599–610. [CrossRef]
17. Velnar, T.; Bailey, T.; Smrkolj, V. The Wound Healing Process: An Overview of the Cellular and Molecular Mechanisms. *J. Int. Med. Res.* **2009**, *37*, 1528–1542. [CrossRef]
18. Gonzalez, A.C.d.O.; Costa, T.F.; Andrade, Z.d.A.; Medrado,, A.R.A.P. Wound Healing—A Literature Review. *An. Bras. De Dermatol.* **2016**, *91*, 614–620. [CrossRef]
19. Sharma, A.; Puri, V.; Kumar, P.; Singh, I. Biopolymeric, Nanopatterned, Fibrous Carriers for Wound Healing Applications. *Curr. Pharm. Des.* **2020**, *26*, 4894–4908. [CrossRef]
20. Darby, I.A.; Laverdet, B.; Bonté, F.; Desmoulière, A. Fibroblasts and Myofibroblasts in Wound Healing. *Clin. Cosmet. Investig. Dermatol.* **2014**, *7*, 301–311. [CrossRef]
21. Young, A.; McNaught, C.E. The Physiology of Wound Healing. *Surg. (United Kingd.)* **2011**, *29*, 475–479. [CrossRef]
22. Marume, A.; Matope, G.; Katsande, S.; Khoza, S.; Mutingwende, I.; Mduluza, T.; Munodawafa-Taderera, T.; Ndhlala, A.R. Wound Healing Properties of Selected Plants Used in Ethnoveterinary Medicine. *Front. Pharmacol.* **2017**, *8*, 544. [CrossRef] [PubMed]
23. Sen, C.K.; Gordillo, G.M.; Roy, S.; Kirsner, R.; Lambert, L.; Hunt, T.K.; Gottrup, F.; Gurtner, G.C.; Longaker, M.T. Human Skin Wounds: A Major and Snowballing Threat to Public Health and the Economy. *Wound Repair Regen.* **2009**, *17*, 763–771. [CrossRef]
24. Abubakar, K.; Abdulkadir, R.; Ear, N.; Centre, C. Evaluation of the Anticonvulsant Effect of the Methanol Extract of Evolvulus Alsinoides in Mice. *Sch. Acad. J. Pharm.* **2013**, *2*, 436–441.
25. Tyavambiza, C.; Elbagory, A.M.; Madiehe, A.M.; Meyer, M.; Meyer, S.; Elaissari, A. The Antimicrobial and Anti-Inflammatory Effects of Silver Nanoparticles Synthesised from Cotyledon Orbiculata Aqueous Extract. *Nanomaterials* **2021**, *11*, 1343. [CrossRef]

26. Ghaedi, M.; Yousefinejad, M.; Safarpoor, M.; Khafri, H.Z.; Purkait, M.K. Rosmarinus Officinalis Leaf Extract Mediated Green Synthesis of Silver Nanoparticles and Investigation of Its Antimicrobial Properties. *J. Ind. Eng. Chem.* **2015**, *31*, 167–172. [CrossRef]
27. Kumar, V.; Yadav, S.C.; Yadav, S.K. Syzygium Cumini Leaf and Seed Extract Mediated Biosynthesis of Silver Nanoparticles and Their Characterization. *J. Chem. Technol. Biotechnol.* **2010**, *85*, 1301–1309. [CrossRef]
28. Kiran Kumar, H.A.; Mandal, B.K.; Mohan Kumar, K.; Maddinedi, S.B.; Sai Kumar, T.; Madhiyazhagan, P.; Ghosh, A.R. Antimicrobial and Antioxidant Activities of Mimusops Elengi Seed Extract Mediated Isotropic Silver Nanoparticles. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2014**, *130*, 13–18. [CrossRef]
29. Marslin, G.; Siram, K.; Maqbool, Q.; Selvakesavan, R.K.; Kruszka, D.; Kachlicki, P.; Franklin, G. Secondary Metabolites in the Green Synthesis of Metallic Nanoparticles. *Materials* **2018**, *11*, 940. [CrossRef]
30. Shafey, A.M. El Green Synthesis of Metal and Metal Oxide Nanoparticles from Plant Leaf Extracts and Their Applications: A Review. *Green Processing Synth.* **2020**, *9*, 304–339. [CrossRef]
31. Fafal, T.; Taştan, P.; Tüzün, B.S.; Ozyazici, M.; Kivcak, B. Synthesis, Characterization and Studies on Antioxidant Activity of Silver Nanoparticles Using Asphodelus Aestivus Brot. Aerial Part Extract. *South Afr. J. Bot.* **2017**, *112*, 346–353. [CrossRef]
32. Abdel-Aziz, M.S.; Shaheen, M.S.; El-Nekeety, A.A.; Abdel-Wahhab, M.A. Antioxidant and Antibacterial Activity of Silver Nanoparticles Biosynthesized Using Chenopodium Murale Leaf Extract. *J. Saudi Chem. Soc.* **2014**, *18*, 356–363. [CrossRef]
33. Salari, S.; Bahabadi, S.E.; Samzadeh-Kermani, A.; Yosefzaei, F. In-Vitro Evaluation of Antioxidant and Antibacterial Potential of Green Synthesized Silver Nanoparticles Using Prosopis Farcta Fruit Extract. *Iran. J. Pharm. Res.* **2019**, *18*, 430–445. [CrossRef] [PubMed]
34. Swilam, N.; Nematallah, K.A. Polyphenols Profile of Pomegranate Leaves and Their Role in Green Synthesis of Silver Nanoparticles. *Sci. Rep.* **2020**, *10*, 14851. [CrossRef] [PubMed]
35. Ovais, M.; Khalil, A.T.; Islam, N.U.; Ahmad, I.; Ayaz, M.; Saravanan, M.; Shinwari, Z.K.; Mukherjee, S. Role of Plant Phytochemicals and Microbial Enzymes in Biosynthesis of Metallic Nanoparticles. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 6799–6814. [CrossRef] [PubMed]
36. Liang, N.; Kitts, D.D. Antioxidant Property of Coffee Components: Assessment of Methods That Define Mechanisms of Action. *Molecules* **2014**, *19*, 19180–19208. [CrossRef]
37. Badeggi, U.M.; Ismail, E.; Adeloye, A.O.; Botha, S.; Badmus, J.A.; Marnewick, J.L.; Cupido, C.N.; Hussein, A.A. Green Synthesis of Gold Nanoparticles Capped with Procyanidins from Leucosidea Sericea as Potential Antidiabetic and Antioxidant Agents. *Biomolecules* **2020**, *10*, 452. [CrossRef]
38. Xiao, F.; Xu, T.; Lu, B.; Liu, R. Guidelines for Antioxidant Assays for Food Components. *Food Front.* **2020**, *1*, 60–69. [CrossRef]
39. Payne, A.C.; Mazzer, A.; Clarkson, G.J.J.; Taylor, G. Antioxidant Assays—Consistent Findings from FRAP and ORAC Reveal a Negative Impact of Organic Cultivation on Antioxidant Potential in Spinach but Not Watercress or Rocket Leaves. *Food Sci. Nutr.* **2013**, *1*, 439–444. [CrossRef]
40. Tungmunnithum, D.; Thongboonyou, A.; Pholboon, A.; Yangsabai, A. Flavonoids and Other Phenolic Compounds from Medicinal Plants for Pharmaceutical and Medical Aspects: An Overview. *Medicines* **2018**, *5*, 93. [CrossRef]
41. Younis, N.S.; Semary, N.A.E.L.; Mohamed, M.E. Silver Nanoparticles Green Synthesis via Cyanobacterium Phormidium Sp.: Characterization, Wound Healing, Antioxidant, Antibacterial, and Anti-Inflammatory Activities. *Eur. Rev. Med. Pharmacol. Sci.* **2021**, *25*, 3083–3096.
42. Comino-Sanz, I.M.; López-Franco, M.D.; Castro, B.; Pancorbo-Hidalgo, P.L. The Role of Antioxidants on Wound Healing: A Review of the Current Evidence. *J. Clin. Med.* **2021**, *10*, 3558. [CrossRef] [PubMed]
43. Castro, B.; Palomares, T.; Azcoitia, I.; Bastida, F.; Olmo, M.; Soldevilla, J.J.; Alonso-varona, A. Development and Preclinical Evaluation of a New Galactomannan-Based Dressing with Antioxidant Properties for Wound Healing. *Histol. Histopathol.* **2015**, *30*, 1499–1512. [CrossRef] [PubMed]
44. Zanette, C.; Pelin, M.; Crosera, M.; Adami, G.; Bovenzi, M.; Larese, F.F.; Florio, C. Silver Nanoparticles Exert a Long-Lasting Antiproliferative Effect on Human Keratinocyte HaCaT Cell Line. *Toxicol. Vitr.* **2011**, *25*, 1053–1060. [CrossRef] [PubMed]
45. Bolla, S.R.; Mohammed Al-Subaie, A.; Yousuf Al-Jindan, R.; Papayya Balakrishna, J.; Kanchi Ravi, P.; Veeraraghavan, V.P.; Arumugam Pillai, A.; Gollapalli, S.S.R.; Palpath Joseph, J.; Surapaneni, K.M. In Vitro Wound Healing Potency of Methanolic Leaf Extract of Aristolochia Saccata Is Possibly Mediated by Its Stimulatory Effect on Collagen-1 Expression. *Heliyon* **2019**, *5*, e01648. [CrossRef]
46. Liang, C.-C.; Park, A.Y.; Guan, J.-L. In Vitro Scratch Assay: A Convenient and Inexpensive Method for Analysis of Cell Migration in Vitro. *Nat. Protoc.* **2007**, *2*, 329–333. [CrossRef]
47. Vijayakumar, V.; Samal, S.K.; Mohanty, S.; Nayak, S.K. Recent Advancements in Biopolymer and Metal Nanoparticle-Based Materials in Diabetic Wound Healing Management. *Int. J. Biol. Macromol.* **2019**, *122*, 137–148. [CrossRef]
48. Annamalai, P.; Balashanmugam, P.; Kalaichelvan, P. In vitro wound healing and antimicrobial property of cotton fabrics coated optimized silver nanoparticles synthesized using peltophorum pterocarpum leaf extracts. *Asian J. Pharm. Clin. Res.* **2019**, *12*, 216–222. [CrossRef]
49. Ahn, E.Y.; Jin, H.; Park, Y. Assessing the Antioxidant, Cytotoxic, Apoptotic and Wound Healing Properties of Silver Nanoparticles Green-Synthesized by Plant Extracts. *Mater. Sci. Eng. C* **2019**, *101*, 204–216. [CrossRef]

50. Augustine, R.; Hasan, A.; Yadu Nath, V.K.; Thomas, J.; Augustine, A.; Kalarikkal, N.; Moustafa, A.E.A.; Thomas, S. Electrospun Polyvinyl Alcohol Membranes Incorporated with Green Synthesized Silver Nanoparticles for Wound Dressing Applications. *J. Mater. Sci. Mater. Med.* **2018**, *29*, 163. [CrossRef]
51. Chinnasamy, G.; Chandrasekharan, S.; Bhatnagar, S. Biosynthesis of Silver Nanoparticles from *Melia azedarach*: Enhancement of Antibacterial, Wound Healing, Antidiabetic and Antioxidant Activities. *Int. J. Nanomed.* **2019**, *14*, 9823–9836. [CrossRef] [PubMed]
52. Goktas, Z.; Zu, Y.; Abbasi, M.; Galyean, S.; Wu, D.; Fan, Z.; Wang, S. Recent Advances in Nanoencapsulation of Phytochemicals to Combat Obesity and Its Comorbidities. *J. Agric. Food Chem.* **2020**, *68*, 8119–8131. [CrossRef] [PubMed]
53. de Moura Sperotto, N.D.; Steffens, L.; Veríssimo, R.M.; Henn, J.G.; Péres, V.F.; Vianna, P.; Chies, J.A.B.; Roehe, A.; Saffi, J.; Moura, D.J. Wound Healing and Anti-Inflammatory Activities Induced by a Plantago Australis Hydroethanolic Extract Standardized in Verbascoside. *J. Ethnopharmacol.* **2018**, *225*, 178–188. [CrossRef] [PubMed]
54. Syarina, P.N.A.; Karthivashan, G.; Abas, F.; Arulselvan, P.; Fakurazi, S. Wound Healing Potential of Spirulina Platensis Extracts on Human Dermal Fibroblast Cells. *EXCLI J.* **2015**, *14*, 385–393. [CrossRef]
55. Muniandy, K.; Gothai, S.; Tan, W.S.; Kumar, S.S.; Mohd Esa, N.; Chandramohan, G.; Al-Numair, K.S.; Arulselvan, P. In Vitro Wound Healing Potential of Stem Extract of Alternanthera Sessilis. *Evid. -Based Complementary Altern. Med.* **2018**, *2018*, 3142073. [CrossRef]
56. Negahdari, S.; Galehdari, H.; Kesmati, M.; Rezaie, A.; Shariati, G. Wound Healing Activity of Extracts and Formulations of Aloe Vera, Henna, Adiantum Capillus-veneris, and Myrrh on Mouse Dermal Fibroblast Cells. *Int. J. Prev. Med.* **2017**, *8*, 18. [CrossRef]
57. Khosravitabar, F.; Abrishamchi, P.; Bahrami, A.R.; Matin, M.M.; Ejtehadi, H.; Varasteh-Kojourian, M. Enhanced Cutaneous Wound Healing by the Leaf Extract of Achillea Eriophora D.C. Using the in Vitro Scratch Assay. *J. Sci. Islamic Repub. Iran* **2017**, *28*, 305–312.
58. DesJardins-Park, H.E.; Foster, D.S.; Longaker, M.T. Fibroblasts and Wound Healing: An Update. *Regen. Med.* **2018**, *13*, 491–495. [CrossRef]
59. Bainbridge, P.; Healing, W.; Repair, T. Wound Healing and the Role of Fibroblasts. *J. Wound Care* **2013**, *22*, 407–412.
60. Blaha, M.; Nevoral, J.; Prochazka, R. The Serine Protease Inhibitors and SERPINE1/2 Disrupt Prostaglandin E2 Production and Hyaluronic Acid Retention in FSH-Stimulated Pig Cumulus–Oocyte Complexes. *Reprod. Domest. Anim.* **2019**, *54*, 65–68. [CrossRef]
61. Cooper, P.O.; Haas, M.R.; Kumar, S.; Noonepalle, R.; Shook, B.A. Molecular Sciences Dermal Drivers of Injury-Induced Inflammation: Contribution of Adipocytes and Fibroblasts. *Int. J. Mol. Sci.* **2021**, *22*, 1933. [CrossRef] [PubMed]
62. Ridiandries, A.; Tan, J.T.M.; Bursill, C.A. The Role of Chemokines in Wound Healing. *Int. J. Mol. Sci.* **2018**, *19*, 3217. [CrossRef] [PubMed]
63. Krzyszczyk, P.; Schloss, R.; Palmer, A.; Berthiaume, F. The Role of Macrophages in Acute and Chronic Wound Healing and Interventions to Promote Pro-Wound Healing Phenotypes. *Front. Physiol.* **2018**, *9*, 419. [CrossRef] [PubMed]
64. Huang, X.; Liang, X.; Zhou, Y.; Li, H.; Du, H.; Suo, Y.; Liu, W.; Jin, R.; Chai, B.; Duan, R.; et al. Cdh1 Is Identified as a Therapeutic Target for Skin Regeneration after Mechanical Loading. *Int. J. Biol. Sci.* **2021**, *17*, 353–367. [CrossRef]
65. Plichta, J.K.; Radek, K.A. Sugar-Coating Wound Repair: A Review of FGF-10 and Dermatan Sulfate in Wound Healing and Their Potential Application in Burn Wounds. *J. Burn Care Res.* **2012**, *33*, 299–310. [CrossRef]
66. Yamakawa, S.; Hayashida, K. Advances in Surgical Applications of Growth Factors for Wound Healing. *Burn. Trauma* **2019**, *7*, 10. [CrossRef]
67. Cheng, Y.; Li, Y.; Huang, S.; Yu, F.; Bei, Y.; Zhang, Y.; Tang, J.; Huang, Y.; Xiang, Q. Hybrid Freeze-Dried Dressings Composed of Epidermal Growth Factor and Recombinant Human-Like Collagen Enhance Cutaneous Wound Healing in Rats. *Front. Bioeng. Biotechnol.* **2020**, *8*, 742. [CrossRef]
68. Rigo, A.; Gottardi, M.; Zamò, A.; Mauri, P.; Bonifacio, M.; Krampera, M.; Damiani, E.; Pizzolo, G.; Vinante, F. Macrophages May Promote Cancer Growth via a GM-CSF/HB-EGF Paracrine Loop That Is Enhanced by CXCL12. *Mol. Cancer* **2010**, *9*, 273. [CrossRef]
69. Tan, Y.; Qiao, Y.; Chen, Z.; Liu, J.; Guo, Y.; Tran, T.; Tan, K.S.; Wang, D.Y.; Yan, Y. FGF2, an Immunomodulatory Factor in Asthma and Chronic Obstructive Pulmonary Disease (COPD). *Front. Cell Dev. Biol.* **2020**, *8*, 223. [CrossRef]
70. Vinante, F.; Rigo, A. Heparin-Binding Epidermal Growth Factor-like Growth Factor/Diphtheria Toxin Receptor in Normal and Neoplastic Hematopoiesis. *Toxins* **2013**, *5*, 1180–1201. [CrossRef]
71. Paish, H.L.; Kalson, N.S.; Smith, G.R.; del Carpio Pons, A.; Baldock, T.E.; Smith, N.; Swist-Szulik, K.; Weir, D.J.; Bardgett, M.; Deehan, D.J.; et al. Fibroblasts Promote Inflammation and Pain via IL-1α Induction of the Monocyte Chemoattractant Chemokine (C-C Motif) Ligand 2. *Am. J. Pathol.* **2018**, *188*, 696–714. [CrossRef] [PubMed]
72. Kendall, R.T.; Feghali-Bostwick, C.A. Fibroblasts in Fibrosis: Novel Roles and Mediators. *Front. Pharmacol.* **2014**, *5*, 123. [CrossRef] [PubMed]
73. Flavell, S.J.; Hou, T.Z.; Lax, S.; Filer, A.D.; Salmon, M.; Buckley, C.D. Fibroblasts as Novel Therapeutic Targets in Chronic Inflammation. *Br. J. Pharmacol.* **2008**, *153*, 241–246. [CrossRef] [PubMed]
74. Khalili, A.A.; Ahmad, M.R. A Review of Cell Adhesion Studies for Biomedical and Biological Applications. *Int. J. Mol. Sci.* **2015**, *16*, 18149–18184. [CrossRef] [PubMed]
75. Adorno-Cruz, V.; Liu, H. Regulation and Functions of Integrin A2 in Cell Adhesion and Disease. *Genes Dis.* **2019**, *6*, 16–24. [CrossRef]

76. Girgert, R.; Martin, M.; Kruegel, J.; Miosge, N.; Temme, J.; Eckes, B.; Müller, G.-A.; Gross, O. Integrin A2-Deficient Mice Provide Insights into Specific Functions of Collagen Receptors in the Kidney. *Fibrogenesis Tissue Repair* **2010**, *3*, 19. [CrossRef]
77. Houreld, N.N.; Ayuk, S.M.; Abrahamse, H. Cell Adhesion Molecules Are Mediated by Photobiomodulation at 660 Nm in Diabetic Wounded Fibroblast Cells. *Cells* **2018**, *7*, 30. [CrossRef]
78. Laurens, N.; Koolwijk, P.; De Maat, P. Fibrin Structure and Wound Healing. *J. Thromb. Haemost.* **2006**, *4*, 932–939. [CrossRef]
79. Vuga, L.J.; Ben-Yehudah, A.; Kovkarova-Naumovski, E.; Oriss, T.; Gibson, K.F.; Feghali-Bostwick, C.; Kaminski, N. WNT5A Is a Regulator of Fibroblast Proliferation and Resistance to Apoptosis. *Am. J. Respir. Cell Mol. Biol.* **2009**, *41*, 583. [CrossRef]
80. Fathke, C.; Wilson, L.; Shah, K.; Kim, B.; Hocking, A.; Moon, R.; Isik, F. Wnt Signaling Induces Epithelial Differentiation during Cutaneous Wound Healing. *BMC Cell Biol.* **2006**, *7*, 4. [CrossRef]
81. Elsafadi, M.; Manikandan, M.; Almalki, S.; Mahmood, A.; Shinwari, T.; Vishnubalaji, R.; Mobarak, M.; Alfayez, M.; Aldahmash, A.; Kassem, M.; et al. Transgelin Is a Poor Prognostic Factor Associated with Advanced Colorectal Cancer (CRC) Stage Promoting Tumor Growth and Migration in a TGFβ-Dependent Manner. *Cell Death Dis.* **2020**, *11*, 341. [CrossRef] [PubMed]
82. Suresh, R.; Diaz, R.J. The Remodelling of Actin Composition as a Hallmark of Cancer. *Transl. Oncol.* **2021**, *14*, 101051. [CrossRef] [PubMed]
83. Dvorakova, M.; Nenutil, R.; Bouchal, P. Transgelins, Cytoskeletal Proteins Implicated in Different Aspects of Cancer Development. *Expert Rev. Proteom.* **2014**, *11*, 149–165. [CrossRef] [PubMed]
84. Dube, P.; Meyer, S.; Marnewick, J.L. Antimicrobial and Antioxidant Activities of Different Solvent Extracts from Fermented and Green Honeybush (Cyclopia Intermedia) Plant Material. *South Afr. J. Bot.* **2017**, *110*, 184–193. [CrossRef]
85. Oribayo, O.O.; Owolabi, M.A.; Ukpo, G.E.; Shode, F.O. Antioxidant Activity of Some Nigerian Medicinal Plants Used in Malaria Treatment. *Trop. J. Nat. Prod. Res.* **2018**, *2*, 18–22. [CrossRef]
86. Benzie, I.F.F.; Strain, J.J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. *Anal. Biochem.* **1996**, *239*, 70–76. [CrossRef]
87. Goboza, M.; Meyer, M.; Aboua, Y.G.; Oguntibeju, O.O. Molecules In Vitro Antidiabetic and Antioxidant Effects of Different Extracts of Catharanthus Roseus and Its Indole Alkaloid, Vindoline. *Molecules* **2020**, *25*, 5546. [CrossRef]
88. Elbialy, Z.I.; Atiba, A.; Abdelnaby, A.; Al-Hawary, I.I.; Elsheshtawy, A.; El-Serehy, H.A.; Abdel-Daim, M.M.; Fadl, S.E.; Assar, D.H. Collagen Extract Obtained from Nile Tilapia (Oreochromis Niloticus L.) Skin Accelerates Wound Healing in Rat Model via up Regulating VEGF, BFGF, and α-SMA Genes Expression. *BMC Vet. Res.* **2020**, *16*, 352. [CrossRef]

CHAPTER 4

UNPUBLISHED MANUSCRIPT: The cytotoxicity of *Cotyledon orbiculata* aqueous extract and the synthesized biogenic silver nanoparticles

Article

The cytotoxicity of *Cotyledon orbiculata* aqueous extract and the biogenic silver nanoparticles derived from the extract

Caroline Tyavambiza [1, 2], Mervin Meyer [2] and Samantha Meyer [1, *]

[1] Department of Biomedical Sciences, Cape Peninsula University of Technology P.O. Box 1906, Bellville, 7535, Cape Town, South Africa; carolinetyavambiza@gmail.com; meyers@cput.ac.za

[2] DSI/Mintek Nanotechnology Innovation Centre, Department of Biotechnology, University of the Western Cape, Private Bag X17, Bellville 7530, South Africa; memeyer@uwc.ac.za

***** Correspondence: meyers@cput.ac.za; Tel.: +27-21-959-6251

Abstract

Silver nanoparticles (AgNPs) have become popular because of their antimicrobial activities. Plants have been used to synthesize AgNPs in a bid to produce nanoparticles that are safer for biomedical applications. Unlike in chemical nanoparticle synthesis, green synthesis does not involve the use of hazardous chemicals. In a previous study we successfully synthesized AgNPs (*Cotyledon*-AgNPs) using an extract of *Cotyledon orbiculata,* a medicinal plant traditionally used in South Africa to treat skin conditions. *Cotyledon*-AgNPs were also shown to have significant antimicrobial activity. Epithelial, fibroblast and keratinocyte cell lines treated with extracts of *C. orbiculata* and *Cotyledon*-AgNPs demonstrated an enhanced growth rate. These nanoparticles therefore display promising wound healing activities. However, the cytotoxicity of these nanoparticles is not known. In this study, the toxic effects of *C. orbiculata* extract and *Cotyledon*-AgNPs to the non-cancerous skin fibroblast *(*KMST-6), keratinocyte (HaCaT) and epithelial (CHO) cell lines were determined using in vitro assays. The assays used in this study included the Mitochondrial Membrane Potential Assay, APOPercentage^{TM} assay, and reactive oxygen species assay. The *C. orbiculata* extract (15 µg/ml) and *Cotyledon*-AgNPs (2.5 µg/ml) did not show any significant cytotoxic effects at the concentrations tested. Gene expression analysis was also used to assess the cytotoxic effects of *Cotyledon*-AgNPs at a molecular level. The upregulated genes (FASN, SREBF1, CPT2, ASB1, HSPA1B, ABCC2, CASP9 and MKI67) supported the finding that *Cotyledon*-AgNPs have low cytotoxicity at the concentrations tested. The gene expression analysis did however support previous findings that *Cotyledon*-AgNP-

treatment promotes cell proliferation as shown by the upregulation of FASN, SERBF1, MKI-67 and HSPA1B. It can be concluded that *Cotyledon*-AgNPs are not toxic to the skin fibroblast cells at the concentration used in this study and that these nanoparticles promote cell growth and could possibly be used for wound healing.

Keywords:

Green nanotechnology; Silver nanoparticles; *Cotyledon Orbiculata;* Cell toxicity; In-vitro assays

1. Introduction

Silver has a long history of use in the medicinal field. Its use has been recorded from as early as 4000 B.C.E by the ancient Greeks, Romans, and Egyptians [1]. It was used to make different silver configurations such as plates, cups, containers and other utensils [2]. It was believed that the use of silver utensils could preserve food and water and could also prevent people from getting infectious diseases [1,2]. After silver was recognized to have antimicrobial activity, it was also incorporated in many other aspects of medicine. It was used for the treatment of infected wounds, burn wounds, skin ulceration and to prevent gonococcal ophthalmic infections in new-born babies [3]. Silver has also been used as a coating for medical catheters (urinary, venous, drainage catheters), surgical blades and needles in order to prevent infections [4]. Due to technological advancement and the emergence of nanotechnology, the synthesis of silver nanoparticles has become popular. Silver nanoparticles are known to exert greater antimicrobial activities than the silver metal because of their smaller size and higher surface area [2,5]. They were reported to be effective against drug resistant microorganisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and ampicillin-resistant *Escherichia coli* [6]. Because of their properties, AgNPs are also applied in food preservation and packaging materials, water treatment, cosmetics, clothing & textiles, biosensing and imaging [7].

Prolonged use of high doses of silver is however not recommended. Prolonged use of silver has been associated with agyria, a condition in which silver deposition in normal skin and tissues causes discoloration. However, chronic argyria does not cause any physiological or pathologic alterations, it is only cosmetically undesirable [1,2]. The toxicity of AgNPs has also been

reported. Nanoparticles exert their toxicity through reactive oxygen species (ROS) generation, mitochondrial dysfunction, membrane damage and protein oxidation. Their toxicity is determined by their characteristics which include shape, size, concentration and surface coating [8–10]. Chemically synthesized nanoparticles have been associated with toxicity mostly because of the way they are synthesized. The chemical synthesis of AgNPs involves the use of toxic chemicals such as sodium borohydride and sodium citrate [11,12]. To reduce toxicity of nanomaterial, researchers and scientists have turned to green nanotechnology, a field in which nanomaterials are synthesized using biomaterials such as plants and microorganisms instead of the hazardous chemicals. Plant extracts are used as both reducing and capping agents in the nanoparticle synthesis [12]. Even though green nanoparticles are expected to be safer than the chemical ones, some studies state that toxicity is rendered by both the AgNP and the silver ion [10,13], therefore green nanoparticles may still be toxic to some extent. It is therefore important to determine the toxicity of the synthesized green nanoparticles before use.

C. orbiculata, a medicinal plant indigenous to South Africa was successfully used to synthesize silver nanoparticles [14]. The synthesized silver nanoparticles (*Cotyledon*-AgNPs) with a size of 40-60 nm, exhibited good antimicrobial, anti-inflammatory [14] and wound healing properties [15]. Their antimicrobial activity was comparable to and in some instances better than the activity of commercial antimicrobial drugs, ampicillin, and fluconazole [14]. The *C. orbiculata* plant was traditionally used to treat skin wounds, boils and acne [16]. In our previous study, the *C. orbiculata* extract and the *Cotyledon*-AgNPs increased the growth of HaCaT, KMST-6 and CHO cells at low concentrations of 15 and 2.5 µg/ml respectively. At these concentrations, they also showed some wound healing activities as they closed the scratched gap faster than untreated cells in wound healing scratch assays. Gene expression studies using a wound healing gene panel showed that the *Cotyledon*-AgNPs and the *C. orbiculata* extracts promote keratinocyte and fibroblast proliferation and migration by upregulating genes such as FGF7 and FGF10. They also upregulated genes involved in collagen construction, ECM formation, cell adhesion and cytoskeleton organization (COL5A3, COL14A1, ITGB1, ITGB6, ACTA1 and TAGLN) [15]. *Cotyledon*-AgNPs can thus be used as potential wound healing agents. It is therefore important to evaluate their potential cytotoxic effects. Thus, the aim of this study was to evaluate the toxicity of *Cotyledon*-AgNPs in KMST-6 cells. This was done by determining the effects of the *Cotyledon*-AgNPs on ROS production, mitochondrial membrane potential and apoptosis in these cells. Considering the limitations of bioassays in studying the effects of

nanomaterials, the study also used gene expression analysis to assess the effects of the *Cotyledon*-AgNPs on the expression levels of genes involved in toxicity.

2. Results and discussion

2.1. Synthesis of Cotyledon-AgNPs

The *Cotyledon*-AgNPs were successfully synthesized and characterised using UV-Vis, dynamic light scattering (DLS) and High resolution transmission electron microscopy (HR-TEM) following the procedure reported in our previous study [14].

2.2. The cytotoxicity of C. orbiculata extracts and Cotyledon-AgNPs

To assess the cytotoxic effects of the *C. orbiculata* extracts and *Cotyledon*-AgNPs, their toxicity was evaluated in KMST-6, HaCaT and CHO cell cultures. The IC_{50} values represent the concentration of a compound that inhibits the viability of cells by 50 % [17,18]. The *C. orbiculata* extracts had higher IC_{50} values compared to the *Cotyledon*-AgNPs and is therefore less toxic than the *Cotyledon*-AgNPs synthesized from it. It can be concluded that the toxicity levels of *Cotyledon*-AgNPs varies in different cell lines, as shown by the different IC_{50} values in KMST-6, HaCaT and CHO cells (Table 1). In this study, the concentrations, 15 and 2.5 µg/ml were used for the *C. orbiculata* extract and *Cotyledon*-AgNPs respectively. These are the concentrations at which the *C. orbiculata* extract and the *Cotyledon*-AgNPs were shown to exhibit cell growth and wound healing activities [14]. As shown in Table 1, these concentrations were much lower than the IC_{50} values.

Table 1. IC_{50} values of *C. orbiculata* extract and the *Cotyledon*-AgNPs in cell cultures

	IC_{50} (µg/ml)		
Treatment	**KMST-6**	**HaCaT**	**CHO**
C. orbiculata extract	296 ± 27.34	>1000	>1000
Cotyledon-AgNPs	122 ± 19.30	40.55 ± 2.68	21.08 ± 0.76

2.3. Cotyledon-AgNPs uptake by KMST-6 cells

For nanoparticles to effectively execute their functional activities, they must be taken up by the cells or be adsorbed on their cell membranes. Nanoparticles can enter the body through ingestion, inhalation or skin contact [19,20]. Using these routes, the nanoparticles can reach

different organs of the body, such as the liver, kidney, lung, spleen, and skin [7,21]. They enter the cells through diffusion or endocytosis [22]. In vitro studies have confirmed the uptake of nanoparticles by various cells such as keratinocytes [23–25], fibroblasts [26], macrophages [27], mesenchymal [23,28], lung [21,29,30], and liver cells [31].

Cellular uptake of *Cotyledon*-AgNPs by skin fibroblasts (KMST-6 cells) was determined using microscopy, both fluorescence and dark field microscopy. A fluorescent dye (DAPI) was used to stain the nuclei. It was shown that after a 24 h incubation with *Cotyledon*-AgNPs, KMST-6 cells successfully take up the *Cotyledon*-AgNPs into their cytoplasm (Figure 1). The uptake of AgNPs using dark field microscopy has been previously reported. Zucker et al. (2019) showed the uptake of branched Polyethyleneimine and citrate coated AgNPs by ARPE-19 cells using dark field microscopy [32].

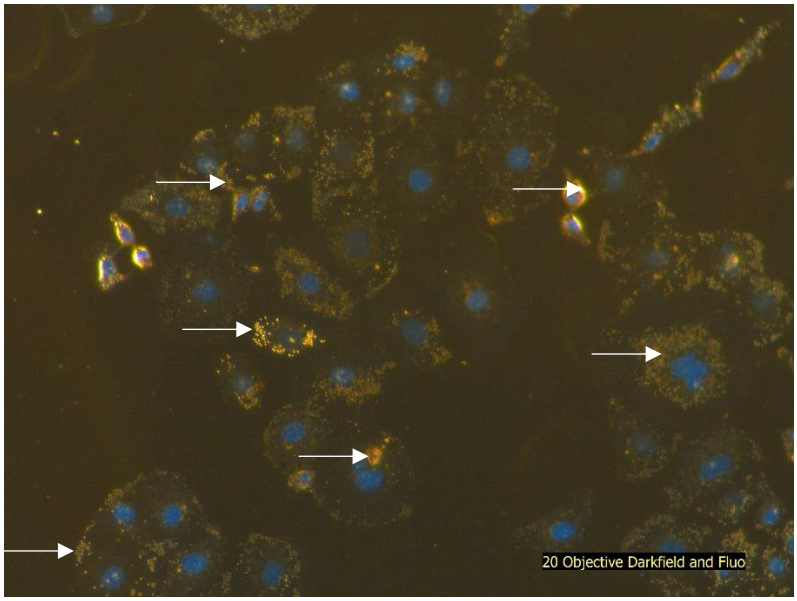


Figure 1. Combined dark field and fluorescence microscopy showing the uptake of *Cotyledon*-AgNPs, 2.5 µg/ml (indicated by white arrows) by KMST-6 cells. The nucleus has been stained by a fluorescent dye, DAPI in blue.

The uptake of nanoparticles by cells depends on several factors which include size, shape and surface charge of the nanoparticles. Many studies have reported the effects of these parameters on cellular uptake. It has been reported that spherical nanoparticles have a higher uptake than asymmetrically shaped ones (nanorods and nanostars) [19,23,31], also positively

charged (cationic) nanoparticles are more efficiently internalized than the neutral and negatively charged nanoparticles [19,33].

Size is also crucial for nanoparticles uptake, studies have shown that smaller nanoparticles are internalized better than the larger ones [34,35]. This might be because cell membranes allow free diffusion of small molecules and particles sized around 10–30 nm [19]. Some nanoparticles are taken into the cells through mechanisms of endocytosis (receptor mediated endocytosis, clathrin mediated endocytosis, caveoli mediated endocytosis, non-clathrin- and non-caveolin-mediated endocytosis and micropinocytosis) [19,36]. Endocytosis can be divided into phagocytosis (which involves the engulfment of particulate matter through cell receptors) and pinocytosis (which involves the internalization of fluids and molecules by small vesicles) [36,37]. In a study by AshaRani et al. (2009), endosomes containing nanoparticles were observed in the cell cytoplasm and near the nuclear membrane, suggesting that the nanoparticles were internalized through endocytosis rather than diffusion [26]. In this study however, in Figure 1, even though microscopy confirmed the presence of nanoparticles in the cell cytoplasm, further studies which may include HR-TEM analysis can be done in the future to confirm the mechanism of nanoparticles internalization.

2.4. Effects of C. orbiculata extracts and Cotyledon-AgNPs on cellular ROS levels

After successful uptake of the nanoparticles, they are transported to different compartments of the cell. Some nanoparticles are found in the cytoplasm, both in the cytosol and in organelles such as the lysosomes and mitochondria while others may penetrate the nucleus [38,39]. Nanoparticles have been reported to increase ROS levels inside the cells leading to toxicity. Actually, ROS production is said to be the most common mechanism of cellular toxicity by nanoparticles [8]. ROS are reactive chemicals that contain oxygen [40]. These by-products of oxygen metabolism include hydrogen peroxide, superoxide anion radicals, and hydroxyl radicals. Under normal conditions, ROS plays an important role in various cellular signaling pathways including growth regulation [41], however increased levels of ROS may have detrimental effects on the cells. Excessive ROS can damage the cellular antioxidant defense systems by increasing oxidative stress while reducing the amounts of glutathione and superoxide dismutase enzymes [21]. ROS generation also affects redox homeostasis causing lipid peroxidation and protein carbonylation. This leads to the damage of DNA, proteins, and lipids eventually causing apoptosis [8]. Nanoparticles have been shown to increase ROS levels by disrupting the electron transfer process, disturbing mitochondrial function and interfering with

the expression of genes involved in oxidative stress [40,42]. The toxicity of AgNPs has been attributed to the particle itself and Ag ionic species that may be released from the nanoparticle [21,30,43,44].

The CM-H_2DCFDA oxidative stress probe was used to determine the effects of *C. orbiculata* extract and *Cotyledon*-AgNPs on ROS production in keratinocytes, fibroblasts and epithelial cells. Both the *C. orbiculata* extract and the Cotyledon-AgNPs did not induce any significant increase in ROS levels in all the tested cell lines (Figure 2). The activity of *Cotyledon*-AgNPs was similar to that of the *C. orbiculata* extract especially in the fibroblast (KMST-6) and epithelial (CHO) cell lines. Interestingly the percentage of CHO cells with increased levels of ROS was lower for cells treated with the *C. orbiculata* extract and *Cotyledon*-AgNPs when compared to the untreated cells, this might be displaying some antioxidant activity. Interestingly, the *C. orbiculata* extracts, but not the *Cotyledon*-AgNPs had a similar effect on HaCaT cells. Similarly, in a study by Gliga et al. (2014), PVP and citrate coated AgNPs did not induce any significant ROS increase in non-cancerous bronchial epithelial cells (BEAS-2B) [30]. However only a few studies have shown results similar to this, many other studies have reported AgNPs to increase intracellular ROS levels in cells including A549 [21,43] and Hep G2 cells [44].

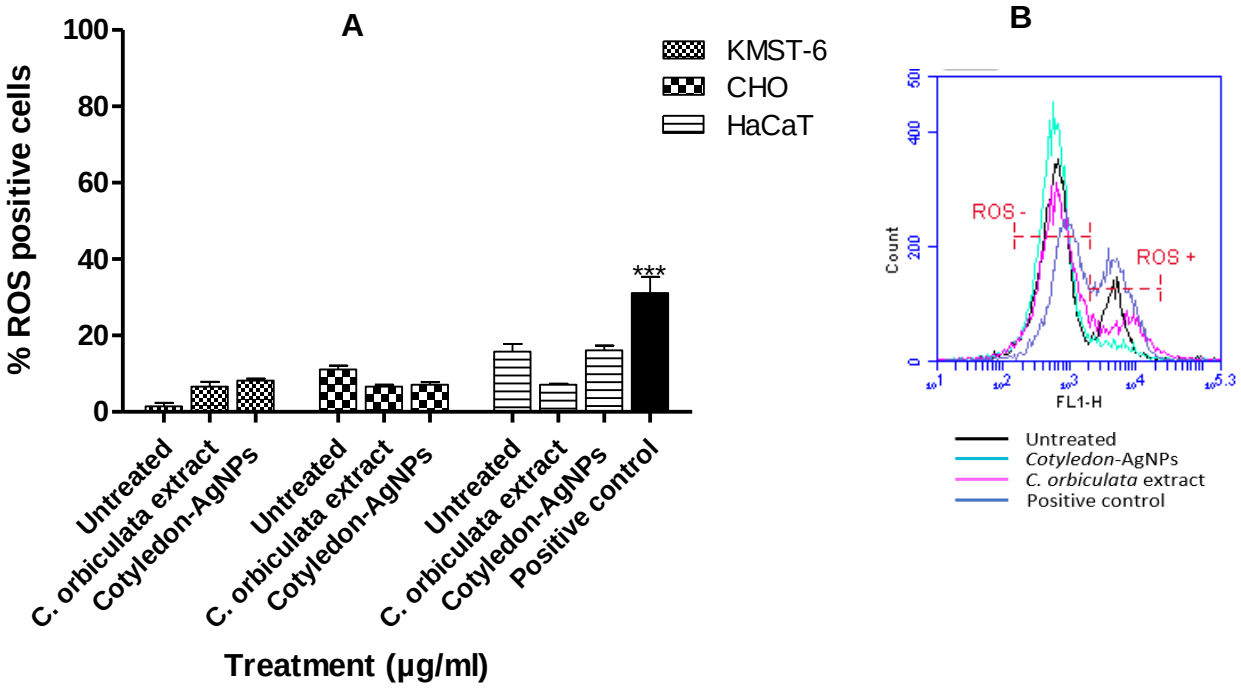


Figure 2. A shows the effects of *Cotyledon*-AgNPs (2.5 µg/ml) and *C. orbiculata* extract (15 µg/ml) on ROS levels in KMST-6, CHO and HaCaT cells. **B** shows an example of a histogram plot of CM-H_2DCFDA probe fluorescence that was generated by flow cytometry for KMST-6 cells. Indicated in the histogram are populations that are positive (ROS+) and negative for (ROS-) for CM-H_2DCFDA fluorescence. Each value represents mean ± standard error of the mean (SEM); statistical significance of the *C. orbiculata* extract and *Cotyledon*-AgNPs-treated cells when compared to the untreated cells is indicated with * for $p < 0.05$, **p for <0.01, *** p <0.001 and **** for $p < 0.0001$.

2.5. Effects of C. orbiculata extracts and Cotyledon-AgNPs on mitochondrial membrane potential

The toxicity of AgNPs can also be determined by investigating their effects on the mitochondria, more specifically the mitochondrial membrane potential (MMP). Mitochondria are essential organelles of the cell that are involved in cellular processes such as cell signaling, cell differentiation, and cell growth [45]. However, the main function of the mitochondria is to produce energy in the form of ATP through the electron transport chain, a process called oxidative phosphorylation [46]. During oxidative phosphorylation the mitochondria produces a significant amount of ROS. This mitochondrially-generated ROS and the MMP are part of the mitochondrial signaling factors [45]. MMP is therefore a good indicator of mitochondrial activity and cell viability [47].

AgNPs have been reported to cause mitochondrial damage either directly through contact or indirectly through mitochondrial membrane depolarization, altered membrane permeability and increased ROS production [48–51]. The effects of *Cotyledon*-AgNPs and *C. orbiculata* extract on MMP were determined using the TMRE assay. This method is based on the ability of the TMRE dye to accumulate in active mitochondria due to their relative negative charge. In depolarized mitochondria, the MMP decreases thus fail to retain the TMRE dye [52]. As shown in Figure 3, cells treated with the *Cotyledon*-AgNPs or *C. orbiculata* extract retained the TMRE dye, indicating that the mitochondria were active in these cells and that there was no significant mitochondrial membrane depolarization. Both treatments showed a similar response to that of the untreated control. These results support the findings of the oxidative stress tests in Figure 2, as mitochondrial membrane depolarization would have led to increased ROS levels and vice versa.

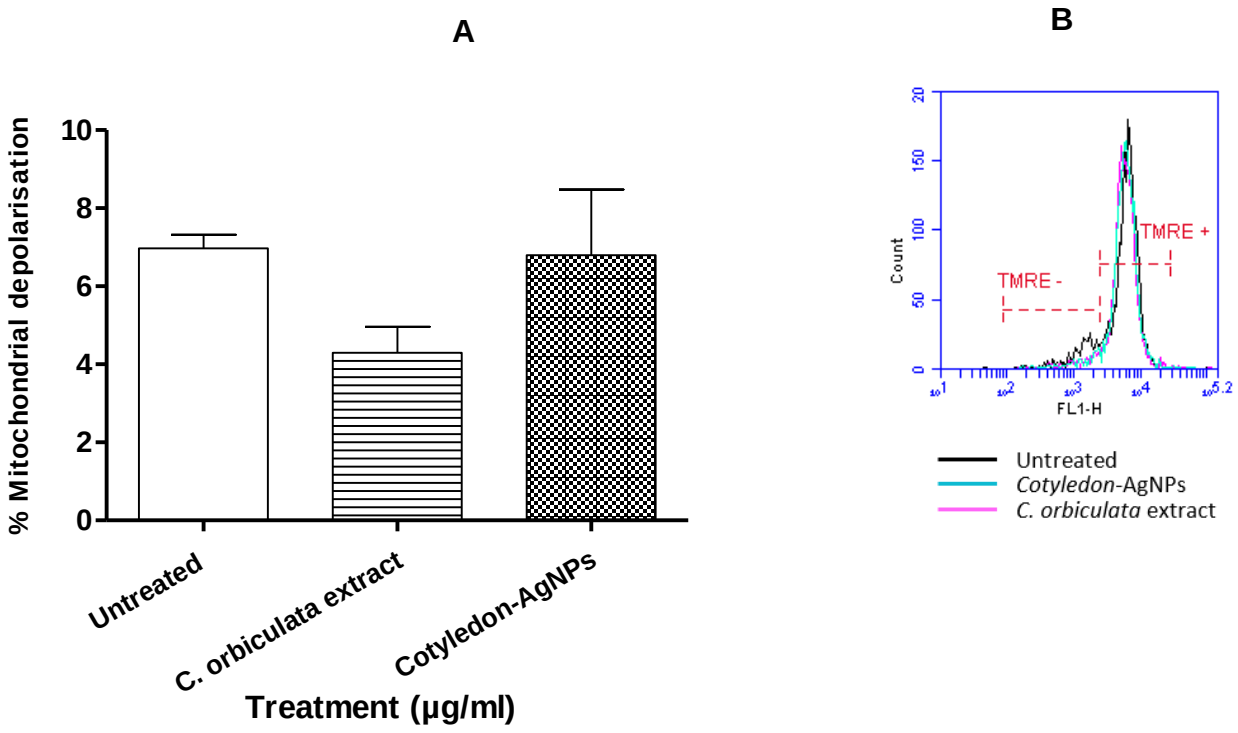


Figure 3. A shows the effects of *Cotyledon*-AgNPs (2.5 µg/ml) and *C. orbiculata* extract (15 µg/ml) on the MMP of KMST-6 cells. Mitochondrial membrane depolarization results in loss of TMRE probe retention and is represented by reduced fluorescence. **B** shows an example of a histogram plot of TMRE fluorescence for KMST-6 cells that was generated flow cytometry. Cell polpulations that are positive (TMRE+) and negative (TMRE-) for TMRE staining are indicated on the plot. Each value represents mean ± SEM; statistical significance of the *C. orbiculata* extract and *Cotyledon*-AgNPs-treated cells when compared to the untreated cells is indicated with * for p <0.05, **p for <0.01, *** p <0.001 and **** for p < 0.0001.

The maintenance of MMP is crucial for the proper functioning of the mitochondria and the health of cells [50]. Low MMP levels, reduce ATP levels eventually causing intracellular acidosis [53]. Acidosis leads to unwanted activation of proteases, nucleases and lipases causing degradation of cellular components and cell death [47]. On the other hand, increased MMP levels increase oxidative phosphorylation thus increasing ROS levels [54]. ROS accumulation further causes mitochondrial membrane depolarization leading to damage of mitochondrial components (DNA, protein and lipids) and loss of MMP, eventually leading to cell death [21,49,55].

2.6. Effects of C. orbiculata extracts and Cotyledon-AgNPs on apoptosis

Apoptosis is a programmed cell death process that can be induced by intracellular or extracellular signals [56]. Nanoparticles have been reported to cause mitochondrial-dependent apoptosis through increased ROS levels and membrane damage [57,58]. Loss of the MMP and impairment of the membrane permeability leads to the release of proapoptotic proteins such as cytochrome c into the cytosol [59]. The released cytochrome c activates caspase 9 which will in-turn activate caspase 3 [45]. Caspase 3 cleaves nuclear DNA causing DNA fragmentation and eventually cell death [56]. Studies have also shown that nanoparticles induce apoptosis through activation of the P53 pathway [60]. P53 enhances the expression of proapoptotic proteins while interacting with and neutralizing the antiapoptotic proteins, therefore causing apoptosis [58,61]. The APOPercentage assay was used to determine the effects of *Cotyledon*-AgNPs and *C. orbiculata* extract on apoptosis in KMST-6 cells. It was shown that both the *C. orbiculata* extract and the *Cotyledon*-AgNPs did not have significant apoptotic effects on the cells as the recorded levels of apoptosis in the treated cells was not significantly higher than that obtained in the untreated cells.

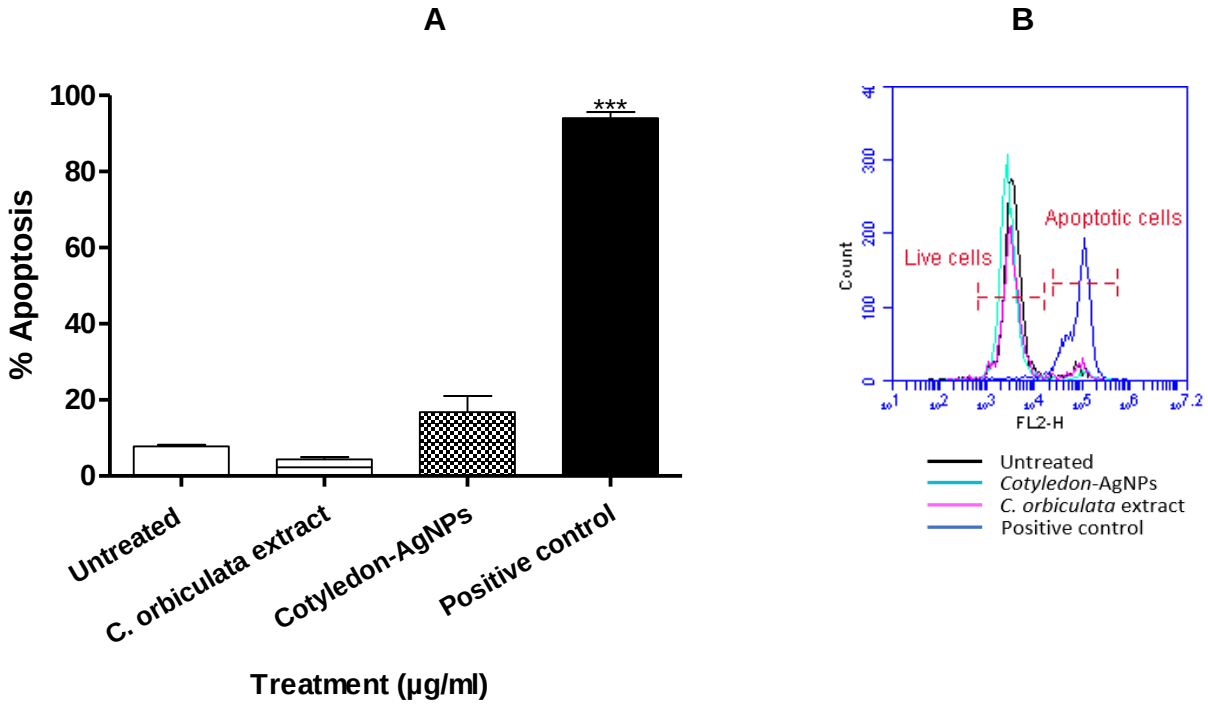


Figure 4. A shows the apoptotic effects of *Cotyledon*-AgNPs (2.5 µg/ml) and *C. orbiculata* extract (15 µg/ml) on KMST-6 cells. **B** shows an example of a histogram plot of the APOPercentage probe fluorescence that was generated by flow cytometry for KMST-6 cells.

The histogram indicates live cell populations (do not take up the APOPercentage dye) and apoptotic cell populations (takes up the APOPercentage dye). Each value represents mean ± SEM; statistical significance of the *C. orbiculata* extract and *Cotyledon-AgNPs* treated cells when compared to the untreated cells is indicated with * for $p < 0.05$, **p for <0.01, *** $p < 0.001$ and **** for $p < 0.0001$.

2.7. Effects of Cotyledon-AgNPs on the expression genes involved in toxicity

Studying the toxicity of nanomaterials using traditional bioassays was reported to have several disadvantages such as producing irreplicable results [62]. However, gene expression technologies are considered one of the best ways to evaluate nanomaterial toxicity [63,64]. Therefore, in this study, gene expression studies were done to determine the molecular effects of *Cotyledon*-AgNPs on a non-cancerous skin fibroblast cell line, KMST-6. A molecular toxicity panel (Human Molecular Toxicology PathwayFinder RT2 Profiler PCR Array) consisting of 84 genes was used for this analysis. A total number of 8 (FASN, SREBF1, CPT2, ASB1, HSPA1B, ABCC2, CASP9 and MKI67) of these 84 genes were differentially expressed. All the differentially expressed genes were upregulated and are shown in Table 2 and in Figure 5 below. Their expression was between 1.5 and 2.5 times higher in the *Cotyledon*-AgNPs treated cells when compared to the untreated cells.

Table 2. Upregulated toxicity genes in *Cotyledon*-AgNPs treated cells

Gene name	Symbol	Function
Fatty acid synthase	FASN	Steatosis
Sterol regulatory element-binding transcription factor 1	SREBF1	Steatosis
Carnitine palmitoyl transferase II	CPT2	Fatty Acid Metabolism (β-Oxidation)
Ankyrin repeat and SOCS box protein 1	ASB1	Mitochondrial Energy Metabolism
Heat Shock Protein Family A (Hsp70) Member 1B	HSPA1B	Mitochondrial Energy Metabolism
ATP Binding Cassette Subfamily C Member 2	ABCC2	Cholestasis
Caspase-9	CASP9	Apoptosis
Marker Of Proliferation Ki-67	MKI67	Immunotoxicity

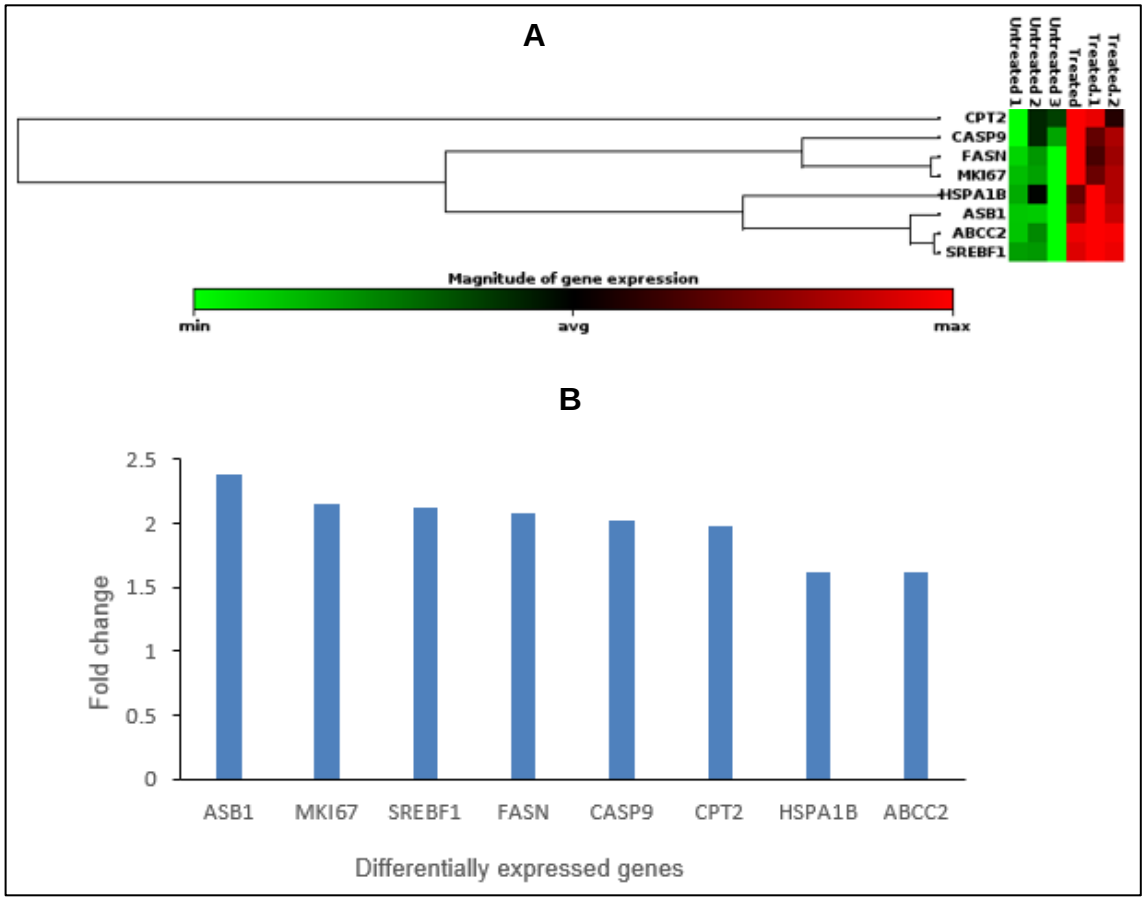


Figure 5. A is a clustergram of the upregulated genes in the *Cotyledon*-AgNPs (2.5 µg/ml) treated KMST-6 cells. **B** is a bar chart showing the fold changes of the DEGs, genes with p values <0.05 and absolute fold change ≥1.5 were considered differentially regulated.

STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) was used to investigate whether these genes are part of any functional protein-protein networks. This analysis showed that the upregulated genes clustered into 2 groups while some of the genes did not form part of any known functional network (Figure 6). STRING analysis demonstrated functional networks between 3 (FASN, CPT2 and SREBF1) of 8 genes, while another network existed for 2 other genes (MKI-67 and CASP9). FASN, CPT2 and SREBF1 are involved in lipid metabolism, oxidation and haemostasis respectively. FASN is involved in fatty acid metabolism, it catalyses the formation of the long chain saturated fatty acid, palmitate from acetyl-CoA and malonyl-CoA [65,66]. The palmitate leads to the production of different lipids including phospholipids that are used in the formation of membranes. FASN has mostly been associated with cancer cell proliferation, however a study by Veigel et al. (2015) showed that FASN promoted the growth of normal ovarian epithelial cells [67]. Because of their findings, they labelled FASN as a marker of cell proliferation rather than that of cancer growth. Our findings support the notion of FASN as a cell proliferation marker.

In our previous study it was shown that *Cotyledon*-AgNPs promote cell growth and wound healing [15], the upregulation of FASN therefore supports this finding. The gene SERBF1 which controls genes that are involved in lipid synthesis (e.g. FASN) in-order to maintain cellular lipid homoeostasis [64,68,69] was also upregulated. SREBFs have been shown to connect lipid metabolism with nutrition and cell growth. In response to low cholesterol levels, SREBF1 moves from the endoplasmic reticulum to the Golgi apparatus and eventually the nucleus, where it induce the expression of genes involved in lipid synthesis [69]. SREBF1 mostly regulates the expression of factors required for fatty-acid synthesis, while SREBF2 regulates those for cholesterol synthesis [68]. Because the body needs to maintain homeostasis, any action in the body has a counteraction. In this case, increased lipid production by the *Cotyledon*-AgNPs treated cells possibly led to the upregulation of the CPT2 gene. CPT2 is found on the inner membrane of the mitochondria, where it is involved in fatty acid oxidation and in preserving the structure of the mitochondria [70,71]. Fatty acid oxidation is the breakdown of fatty acids into acetyl-CoA, while producing ATP and NAPDH. NAPDH provides the reducing power for anabolic reactions and also counteract oxidative stress [71,72]. CPT2 might have contributed to reduced cellular ROS levels in *Cotyledon*-AgNPs treated HaCaT, CHO and KMST-6 cells (Figure 2).

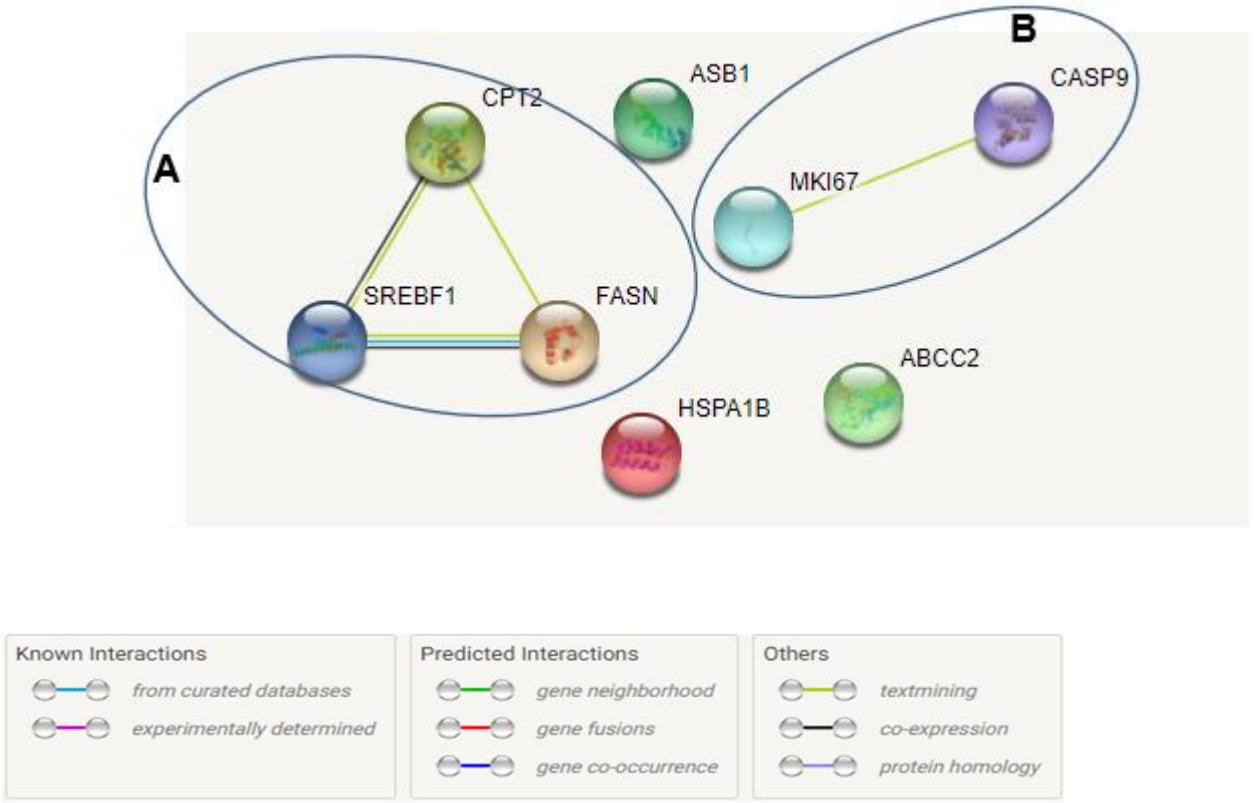


Figure 6. Protein networks showing the interactions between the DEGs. These networks were determined using the STRING database.

The observed upregulation of the gene MKI-67 in cluster B correlates with the upregulation of the gene, FASN. MKI-67 is a proliferation marker, it is expressed in proliferating cells and absent in resting cells. The detection of MKI-67 has been used as an indicator for proliferating cells [73]. Interestingly in this cluster, CASP9, a gene with effects opposite to the MKI-67 was also upregulated. CASP9 is an apoptotic gene which is involved in the activation of caspases responsible for apoptosis. After being activated by binding to Apaf-1, CASP9 activates other caspases including Caspase 3 and 7 [74,75]. The upregulation of the CASP9 was probably the counteraction by the cells as they were trying to return to haemostasis after increased cellular proliferation. Even though CASP9 was upregulated, it did not induce apoptosis of the *Cotyledon*-AgNPs treated fibroblasts as shown by the APOPercentage assay (Figure 4) and previous studies which confirm cell growth at the concentrations used in this study [15].

The 3 genes that did not form part of any functional network are ASB1, HSPA1B and ABCC2. ASB1 is a member of the ankyrin repeat and SOCS box-containing (ASB) family and it is mainly involved in the process of ubiquitination which includes protein modification or misfolding which occur because of cell damage. ASB1 is also associated with the expression of proinflammatory genes [76]. The effects of ASB1 might however not be expressed because of the upregulation of the HSPA1B gene. The HSPA1B gene encodes a member of heat shock proteins found in the cytoplasm. This group of genes protect cells from a range of stresses including proteotoxic stress by identifying and repairing misfolded or damaged proteins and preventing the aggregation of modified proteins [77,78]. Heat shock proteins (HSPs) are critical for maintaining functional cellular pathways, protecting cell integrity and ultimately promoting cell survival [77,78]. Hsp70 proteins have also been reported to inhibit caspase-dependent and caspase-independent apoptosis by neutralizing apoptosis-inducing factor and also inhibiting the binding of Apaf1 to procaspase-9 thus preventing its activation [79]. This is probably why no apoptotic effects were seen in the cells (Figure 4).

The third gene that was upregulated was ABCC2, a member of the ATP binding cassette (ABC) family. As part of the ABC transport proteins, ABCC2 transports various compounds across the cell membranes and epithelial barriers [80,81]. ABC proteins have been reported to transport different compounds including fatty acids and lipid compounds across membrane barriers [82]. ABCC2 was probably upregulated to transport fatty acids and lipids produced because of the upregulated FASN and SERBF1 genes. The gene panel used in this study investigated the expression of genes involved in oxidative stress, DNA damage and necrosis. The study shows that the expression of these genes was not affected by treatment with *Cotyledon*-AgNPs. This is in agreement with the findings of the various bioassays (ROS, TMRE and APOPercentage) which demonstrated that the nanoparticles are not toxic. In fact, the gene expression study support previous findings [15] which suggest that *Cotyledon*-AgNPs may promote cell growth as shown by the upregulation of FASN, SERBF1, MKI-67 and HSPA1B.

3. Materials and Methods

3.1. Cell culture

HaCaT, KMST-6 and CHO cells were obtained from the DSI/Mintek NIC laboratory at the University of the Western Cape (South Africa). HaCaT and KMST-6 cells were grown in Dulbecco's modified eagle medium (DMEM) while CHO cells were grown in Hams-F12 medium.

Both media was supplemented with 10 % Fetal bovine serum (FBS) and 1 % Pen-strep. All the cells were maintained in a humidified atmosphere of 5 % CO_2 in a 37 °C incubator (SL SHEL LAB, Sheldon manufacturing, Cornelius, OR, USA).

3.2. Determination of IC_{50}

The IC_{50} values of the *C. orbiculata* extract and *Cotyledon*-AgNPs were determined using the WST1 assay. Briefly, cells were seeded in 96 well plates (1×10^5 cell/ml) and incubated for 24 h. After incubation the cells were exposed to different concentrations of the *C. orbiculata* extract and *Cotyledon*-AgNPs for 24 h. The treatments were replaced with 10 % WST-1 reagent diluted in appropriate culture medium. After a 3 h incubation the absorbance was measured at 440 nm (reference 630 nm) using a microplate reader (POLARstar Omega plate reader). The IC_{50} values were calculated using the GraphPad Prism 6 software.

3.3. Cellular uptake of Cotyledon-AgNPs using Dark field microscopy

The cellular uptake of the *Cotyledon*-AgNPs was evaluated using dark field microscopy. Sterile 12 mm round coverslips were placed in the wells of a 6 well culture plate, KMST-6 cells were added to the coverslips and were incubated for 24 h at 37 °C. After incubation, the cells were treated with *Cotyledon*-AgNPs and re-incubated for another 24 h. The cells were then fixed in 4 % paraformaldehyde for 15 min and were afterwards washed in PBS. The coverslips were then inverted onto a clean slide, mounted with the fluorescence dye, DAPI and were viewed under the dark-field microscope (Leica DM2500).

3.4. ROS assay

The levels of ROS were determined by flow cytometry using the cell permeable fluorogenic dye CM-H_2DCFDA. This dye diffuses into cells and is deacetylated to a non-fluorescent compound by intracellular esterases, it is then oxidized by ROS into a highly fluorescent compound dichlorofluorescein (DCF) which can be detected by the flow cytometer. The resulting fluorescence intensity will therefore be proportional to the levels of ROS within the cell. The assay was performed according to a method by [83] with modifications. In brief, KMST-6, CHO and HaCaT cells were seeded in 24 well plates at a density of 1×10^5 cells/ml, at standard culture conditions (5 % CO_2 at 37 °C). After 24 h, the cells were treated with *Cotyledon*-AgNPs and the *C. orbiculata* extract, control cells were left untreated. All the cells were incubated for a

further 24 h at standard conditions. After incubation, the cells were trypsinized, washed with PBS and incubated with 200 µl of diluted DCFDA (7.5 µM) for 30 min at 37 °C in the dark. Following incubation, the DCFDA solution was removed, and the cells were washed with PBS. 200 µl of fresh media was added to the cells and the fluorescence readings were immediately read on a BD Accuri C6 flow cytometer.

3.5. TMRE-Mitochondrial Membrane Potential Assay

Mitochondrial membrane potential was evaluated using the tetramethylrhodamine ethyl ester (TMRE, Sigma) staining method according to the method described by [52] with slight modifications. In short, KMST-6 cells were seeded in 12-well culture plates at a density of 2×10^5 cells/ml. After a 24 h incubation at 37 °C, the cells were treated with *Cotyledon*-AgNPs and the *C. orbiculata* extract, negative control was left untreated. The cells were then trypsinized, centrifuged and resuspended in 250 µl of the TMRE dye (4 µM) diluted in DMEM. The cells were stained for 30 min, washed to remove excess stain, resuspended in 300 µl of PBS and analyzed using the BD Accuri C6 flow cytometer.

3.6. APOPercentage™ assay

The apoptotic effects of the *Cotyledon*-AgNPs were determined using the APOPercentage™ assay (Biocolor Ltd., Carrickfergus, Ireland) following the method by [84]. Briefly, KMST-6 cells were seeded in 12 well plates and incubated for 24 h at 37 °C. The cells were treated with *Cotyledon*-AgNPs, *C. orbiculata* extract and the positive control (H_20_2). The cells were incubated for a further 24 h, trypsinized, centrifuged and stained with 250 µl of the APOP*ercentage*™ dye. After a 30 min incubation with the dye, the stained cells were washed, centrifuged, and resuspended in 300 µl of 1X PBS. Analysis was done using the BD Accuri C6 flow cytometer.

3.7. Gene expression studies using the Human Molecular Toxicology PathwayFinder RT2 Profiler PCR Array

Gene expression studies were done according to the method used by [64]. Briefly, KMST-6 cells were seeded in 25 cm^2 cell culture flasks at 2×10^5 cell/ml. The cells were treated with *Cotyledon*-AgNPs, untreated flasks were used as controls. After a 24 h incubation, the cells were trypsinized, centrifuged and collected in 2 ml Eppendorf tubes. Total RNA extraction was done using the RNeasy Mini Kit (Qiagen, Maryland, USA) according to the manufacturer's

instructions. The RNA concentration and integrity were determined using a Qubit® 2.0 Fluorometer (Invitrogen by Life Technologies, Carlsbad, CA, USA) and 1 % agarose gel electrophoresis respectively. After checking the RNA quality, cDNA synthesis was done using the RT2 First Strand Kit (Qiagen, Maryland, USA). The synthesized cDNA was used for RT-qPCR, which was done using the Human Molecular Toxicology PathwayFinder RT2 Profiler PCR Array (Qiagen, Maryland, USA). The RT-qPCR assay was done on the Roche LightCycler 480 (Roche). Data collected from the LightCycler 480 was analyzed on a Qiagen GeneGlobe Data Analysis Center (https://geneglobe.qiagen.com/za/). The cycle threshold (CT) values were used to determine fold changes. Genes with a fold change of ≥ ± 1.5 and p-values of < 0.05 were considered as differentially expressed genes (DEGs) and were used for further analysis. The Database for Annotation, Visualization and Integrated Discovery (DAVID; version 6.7) and Search Tool for the Retrieval of Interaction Genes/Proteins (STRING; https://string-db.org/) pathways were used to further analyze the different interactions of the DEGs.

4. Conclusions

It can be concluded from the various bioassays used in this study that the *C. orbiculata* extract and *Cotyledon*-AgNPs are not toxic to KMST-6 cells at 15 and 2.5 µg/ml concentrations, respectively and that these treatments are probably also not toxic to CHO and HaCaT cells. Treatments with the *C. orbiculata* extract or *Cotyledon*-AgNPs did not induce oxidative stress, mitochondrial damage or apoptosis. This finding is supported by gene expression analysis which shows that the expression of genes involved in toxicity were largely not affected in KMST-6 cells subjected to *Cotyledon*-AgNPs treatment. Gene expression studies mainly showed the upregulation of genes involved in fatty acid metabolism and mitochondrial energy metabolism. The upregulation of genes involved with lipid metabolism (FASN and SERBF1) and cell proliferation (MKI-67) also support previous findings that *Cotyledon*-AgNPs can promote wound healing by increasing the growth rate of cells involved in would healing such as skin fibroblast cells. Due to the ability of *Cotyledon*-AgNPs to promote the growth of cells involved in wound healing, its low cytotoxicity towards these cells, and its high antimicrobial activity towards microbes that are known to infect wounds, *Cotyledon*-AgNPs can potentially be used as highly effective wound healing agents.

Author Contributions: Conceptualization, M.M. and S.M.; investigation, C.T.; resources, M.M. and S.M.; writing—original draft preparation, C.T.; writing—review and editing, C.T., M.M. and

S.M.; supervision, M.M. and S.M.; funding acquisition, M.M. and S.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the South African National Research Foundation (grant number 114598 and 106642) and the DSI/MINTEK Nanotechnology Innovation Centre (NIC) and “The APC was funded by DSI/MINTEK Nanotechnology Innovation Centre (NIC)”.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Supporting data presented in this study are available on request from the corresponding author.

Acknowledgments: Dr Kirby and the department of IMBM at UWC are highly appreciated for granting us access to the Roche LightCycler 480 instrument.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds used in the study are available on request from the authors.

References

1. Alexander, J.W. History of the Medical Use of Silver *. *surgical infections* **2009**, *10*, 289–292, doi:10.1089/sur.2008.9941.

2. Naik, K.; Kowshik, M. The Silver Lining : Towards the Responsible and Limited Usage of Silver. *Journal of Applied Microbiology* **2017**, *123*, 1068–1087, doi:10.1111/jam.13525.

3. Politano, A.D.; Campbell, K.T.; Rosenberger, L.H.; Sawyer, R.G. Use of Silver in the Prevention and Treatment of Infections : Silver Review. **2013**, *14*, 8–20, doi:10.1089/sur.2011.097.

4. Konop, M.; Damps, T.; Misicka, A.; Rudnicka, L. Certain Aspects of Silver and Silver Nanoparticles in Wound Care: A Minirev...: EBSCOhost. **2016**, *2016*.

5. Ahmed, S.; Ahmad, M.; Swami, B.L.; Ikram, S. A Review on Plants Extract Mediated Synthesis of Silver Nanoparticles for Antimicrobial Applications: A Green Expertise. *Journal of Advanced Research* **2016**, *7*, 17–28, doi:10.1016/J.JARE.2015.02.007.

6. Rai, M.K.; Deshmukh, S.D.; Ingle, A.P.; Gade, A.K. Silver Nanoparticles: The Powerful Nanoweapon against Multidrug-Resistant Bacteria. *Journal of Applied Microbiology* 2012, *112*, 841–852.

7. Zhang, X.; Liu, Z.; Shen, W.; Gurunathan, S. Silver Nanoparticles : Synthesis , Characterization , Properties , Applications , and Therapeutic Approaches. **2016**, doi:10.3390/ijms17091534.

8. Akter, M.; Sikder, M.T.; Rahman, M.M.; Ullah, A.K.M.A.; Hossain, K.F.B.; Banik, S.; Hosokawa, T.; Saito, T.; Kurasaki, M. A Systematic Review on Silver Nanoparticles-Induced Cytotoxicity: Physicochemical Properties and Perspectives. *Journal of Advanced Research* **2018**, *9*, 1–16, doi:10.1016/j.jare.2017.10.008.

9. Zhang, T.; Wang, L.; Chen, Q.; Chen, C. Cytotoxic Potential of Silver Nanoparticles. **2014**, *55*, 283–291.

10. Tortella, G.R.; Rubilar, O.; Durán, N.; Diez, M.C.; Martínez, M.; Parada, J.; Seabra, A.B. Silver Nanoparticles : Toxicity in Model Organisms as an Overview of Its Hazard for Human Health and the Environment. *Journal of Hazardous Materials* **2020**, *390*, 121974, doi:10.1016/j.jhazmat.2019.121974.

11. Rauwel, P.; Küünal, S.; Ferdov, S.; Rauwel, E. A Review on the Green Synthesis of Silver Nanoparticles and Their Morphologies Studied via TEM. **2015**, doi:10.1155/2015/682749.

12. Iravani, S.; Korbekandi, H.; Mirmohammadi, S. V; Zolfaghari, B. Synthesis of Silver Nanoparticles : Chemical , Physical and Biological Methods. **2014**, *9*, 385–406.

13. Ferdous, Z.; Nemmar, A. Health Impact of Silver Nanoparticles: A Review of the Biodistribution and Toxicity Following Various Routes of Exposure. *International Journal of Molecular Sciences* **2020**, *21*, doi:10.3390/IJMS21072375.

14. Tyavambiza, C.; Elbagory, A.M.; Madiehe, A.M.; Meyer, M.; Meyer, S.; Elaissari, A. The Antimicrobial and Anti-Inflammatory Effects of Silver Nanoparticles Synthesised from Cotyledon Orbiculata Aqueous Extract. **2021**, *11*, 1343, doi:10.3390/nano11051343.

15. Tyavambiza, C.; Meyer, M.; Wusu, A.D.; Madiehe, A.M.; Meyer, S. The Antioxidant and in

Vitro Wound Healing Activity of Cotyledon Orbiculata Aqueous Extract and the Synthesized Biogenic Silver Nanoparticles. *Unpublished manuscript* **2022**.

16. Maroyi, A. A Review of Botany, Medicinal Uses, Phytochemistry and Biological Activities of Cotyledon Orbiculata. *Journal of Pharmaceutical Sciences and Research* **2019**, *11*, 3491–3496.

17. Aboyewa, J.A.; Sibuyi, N.R.S.; Meyer, M.; Oguntibeju, O.O. Gold Nanoparticles Synthesized Using Extracts of Cyclopia Intermedia, Commonly Known as Honeybush, Amplify the Cytotoxic Effects of Doxorubicin. *Nanomaterials* **2021**, *11*, doi:10.3390/nano11010132.

18. González-Larraza, P.G.; López-Goerne, T.M.; Padilla-Godínez, F.J.; González-López, M.A.; Hamdan-Partida, A.; Gómez, E. IC50Evaluation of Platinum Nanocatalysts for Cancer Treatment in Fibroblast, HeLa, and DU-145 Cell Lines. *ACS Omega* **2020**, *5*, 25381–25389, doi:10.1021/acsomega.0c03759.

19. Panzarini, E.; Mariano, S.; Carata, E.; Mura, F.; Rossi, M.; Dini, L. Intracellular Transport of Silver and Gold Nanoparticles and Biological Responses: An Update. *International Journal of Molecular Sciences* **2018**, *19*, doi:10.3390/ijms19051305.

20. Dos Santos, C.A.; Seckler, M.M.; Ingle, A.P.; Gupta, I.; Galdiero, S.; Galdiero, M.; Gade, A.; Rai, M. Silver Nanoparticles : Therapeutical Uses , Toxicity , and Safety Issues. *Journal of Pharmaceutical Sciences* **2014**, *103*, 1931–1944, doi:10.1002/jps.24001.

21. Chairuangkitti, P.; Lawanprasert, S.; Roytrakul, S.; Aueviriyavit, S.; Phummiratch, D.; Kulthong, K.; Chanvorachote, P.; Maniratanachote, R. Silver Nanoparticles Induce Toxicity in A549 Cells via ROS-Dependent and ROS-Independent Pathways. *Toxicology in vitro : an international journal published in association with BIBRA* **2013**, *27*, 330–338, doi:10.1016/j.tiv.2012.08.021.

22. McShan, D.; Ray, P.C.; Yu, H. Molecular Toxicity Mechanism of Nanosilver. *Journal of Food and Drug Analysis* **2014**, *22*, 116–127, doi:10.1016/j.jfda.2014.01.010.

23. Graf, C.; Nordmeyer, D.; Sengstock, C.; Ahlberg, S.; Diendorf, J.; Raabe, J.; Epple, M.; Köller, M.; Lademann, J.; Vogt, A.; et al. Shape-Dependent Dissolution and Cellular Uptake of Silver Nanoparticles. *Langmuir* **2018**, *34*, 1506–1519, doi:10.1021/acs.langmuir.7b03126.

24. Vogt, A.; Rancan, F.; Ahlberg, S.; Nazemi, B.; Choe, C.S.; Darvin, M.E.; Hadam, S.; Blume-Peytavi, U.; Loza, K.; Diendorf, J.; et al. Interaction of Dermatologically Relevant Nanoparticles with Skin Cells and Skin. *Beilstein Journal of Nanotechnology* **2014**, *5*, 2363–2373, doi:10.3762/bjnano.5.245.

25. Monteiro-Riviere, N.A.; Samberg, M.E.; Oldenburg, S.J.; Riviere, J.E. Protein Binding Modulates the Cellular Uptake of Silver Nanoparticles into Human Cells: Implications for in Vitro to in Vivo Extrapolations? *Toxicol Lett.* **2013**, *220*, 286–293, doi:10.1016/j.toxlet.2013.04.022.Protein.

26. AshaRani, P. V.; Hande, M.P.; Valiyaveettil, S. Anti-Proliferative Activity of Silver Nanoparticles. *BMC Cell Biology* **2009**, *10*, 65, doi:10.1186/1471-2121-10-65.

27. Singh, R.P.; Ramarao, P. Cellular Uptake, Intracellular Trafficking and Cytotoxicity of Silver Nanoparticles. *Toxicology Letters* **2012**, *213*, 249–259, doi:10.1016/j.toxlet.2012.07.009.

28. Greulich, C.; Diendorf, J.; Simon, T.; Eggeler, G.; Epple, M.; Köller, M. Uptake and Intracellular Distribution of Silver Nanoparticles in Human Mesenchymal Stem Cells. *Acta Biomaterialia* **2011**, *7*, 347–354, doi:10.1016/j.actbio.2010.08.003.

29. Cronholm, P.; Karlsson, H.L.; Hedberg, J.; Lowe, T.A.; Winnberg, L.; Elihn, K.; Wallinder, I.O.; Möller, L. Intracellular Uptake and Toxicity of Ag and CuO Nanoparticles: A Comparison between Nanoparticles and Their Corresponding Metal Ions. *Small* **2013**, *9*, 970–982, doi:10.1002/smll.201201069.

30. Gliga, A.R.; Skoglund, S.; Odnevall Wallinder, I.; Fadeel, B.; Karlsson, H.L. Size-Dependent Cytotoxicity of Silver Nanoparticles in Human Lung Cells: The Role of Cellular Uptake, Agglomeration and Ag Release. *Particle and Fibre Toxicology* **2014**, *11*, 1–17, doi:10.1186/1743-8977-11-11.

31. Lee, Y.J.; Ahn, E.Y.; Park, Y. Shape-Dependent Cytotoxicity and Cellular Uptake of Gold Nanoparticles Synthesized Using Green Tea Extract. *Nanoscale Research Letters* **2019**, *14*, 1–14, doi:10.1186/s11671-019-2967-1.

32. Zucker, R.M.; Ortenzio, J.; Degn, L.L.; Lerner, J.M.; Boyes, W.K. Biophysical Comparison of Four Silver Nanoparticles Coatings Using Microscopy, Hyperspectral Imaging and Flow Cytometry. *PLoS ONE* **2019**, *14*, 1–24, doi:10.1371/journal.pone.0219078.

33. Jiang, Y.; Huo, S.; Mizuhara, T.; Das, R.; Lee, Y.W.; Hou, S.; Moyano, D.F.; Duncan, B.; Liang, X.J.; Rotello, V.M. The Interplay of Size and Surface Functionality on the Cellular Uptake of Sub-10 Nm Gold Nanoparticles. *ACS Nano* **2015**, *9*, 9986–9993, doi:10.1021/acsnano.5b03521.The.

34. Xu, C.; Tung, G.A.; Sun, S. Size and Concentration Effect of Gold Nanoparticles on X-Ray Attenuation As Measured on Computed Tomography. *Chem Mater* **2008**, *20*, 4167–4169, doi:10.1021/cm8008418.Size.

35. Cho, E.C.; Au, L.; Zhang, Q.; Xia, Y. The Effects of Size Shape and Surface Functional Group of Gold Nanostructures on Their Adsorption and Internalization by Cells. *Small* **2010**, *6*, 517–522, doi:10.1002/smll.200901622.The.

36. Mosquera, J.; García, I.; Liz-Marzán, L.M. Cellular Uptake of Nanoparticles versus Small Molecules: A Matter of Size. *Accounts of Chemical Research* **2018**, *51*, 2305–2313, doi:10.1021/acs.accounts.8b00292.

37. Quevedo, A.C.; Lynch, I.; Valsami-Jones, E. Mechanisms of Silver Nanoparticle Uptake by Embryonic Zebrafish Cells. *Nanomaterials* **2021**, *11*, doi:10.3390/nano11102699.

38. Wang, Z.; Li, N.; Zhao, J.; White, J.C.; Qu, P.; Xing, B. CuO Nanoparticle Interaction with Human Epithelial Cells: Cellular Uptake, Location, Export, and Genotoxicity. *Chemical Research in Toxicology* **2012**, *25*, 1512–1521, doi:10.1021/tx3002093.

39. Lankoff, A.; Sandberg, W.J.; Wegierek-Ciuk, A.; Lisowska, H.; Refsnes, M.; Sartowska, B.; Schwarze, P.E.; Meczynska-Wielgosz, S.; Wojewodzka, M.; Kruszewski, M. The Effect of Agglomeration State of Silver and Titanium Dioxide Nanoparticles on Cellular Response of HepG2, A549 and THP-1 Cells. *Toxicology Letters* **2012**, *208*, 197–213, doi:10.1016/j.toxlet.2011.11.006.

40. Yu, Z.; Li, Q.; Wang, J.; Yu, Y.; Wang, Y.; Zhou, Q.; Li, P. Reactive Oxygen Species-Related Nanoparticle Toxicity in the Biomedical Field. *Nanoscale Research Letters* **2020**, *15*, doi:10.1186/s11671-020-03344-7.

41. Fitzmaurice, S.D.; Sivamani, R.K.; Isseroff, R.R. Antioxidant Therapies for Wound Healing: A Clinical Guide to Currently Commercially Available Products. *Skin Pharmacology and Physiology* **2011**, *24*, 113–126, doi:10.1159/000322643.

42. Manke, A.; Wang, L.; Rojanasakul, Y. Mechanisms of Nanoparticle-Induced Oxidative Stress and Toxicity. *BioMed Research International* **2013**, *2013*, doi:10.1155/2013/942916.

43. Beer, C.; Foldbjerg, R.; Hayashi, Y.; Sutherland, D.S.; Autrup, H. Toxicity of Silver Nanoparticles-Nanoparticle or Silver Ion? *Toxicology Letters* **2012**, *208*, 286–292, doi:10.1016/j.toxlet.2011.11.002.

44. Kim, S.; Choi, J.E.; Choi, J.; Chung, K.H.; Park, K.; Yi, J.; Ryu, D.Y. Oxidative Stress-Dependent Toxicity of Silver Nanoparticles in Human Hepatoma Cells. *Toxicology in Vitro* **2009**, *23*, 1076–1084, doi:10.1016/j.tiv.2009.06.001.

45. Tao, M.; You, C.P.; Zhao, R.R.; Liu, S.J.; Zhang, Z.H.; Zhang, C.; Liu, Y. Mitochondria in Human Diseases and Animal Evolution. *Current Molecular Medicine* **2014**, *14*, 115–124.

46. Sack, M.N. Mitochondrial Depolarization and the Role of Uncoupling Proteins in Ischemia Tolerance. *Cardiovascular Research* **2006**, *72*, 210–219, doi:10.1016/j.cardiores.2006.07.010.

47. Zorova, L.D.; Popkov, V.A.; Plotnikov, E.Y.; Silachev, D.N.; Pevzner, I.B.; Jankauskas, S.S.; Babenko, V.A.; Zorov, S.D.; Balakireva, A. V.; Juhaszova, M.; et al. Mitochondrial Membrane Potential. *Analytical Biochemistry* **2018**, *552*, 50–59, doi:10.1016/j.ab.2017.07.009.

48. Bressan, E.; Ferroni, L.; Gardin, C.; Rigo, C.; Stocchero, M.; Vindigni, V.; Cairns, W.; Zavan, B. Silver Nanoparticles and Mitochondrial Interaction. *International Journal of Dentistry* **2013**, *2013*, doi:10.1155/2013/312747.

49. Vuković, B.; Milić, M.; Dobrošević, B.; Milić, M.; Ilić, K.; Pavičić, I.; Šerić, V.; Vrček, I.V. Surface Stabilization Affects Toxicity of Silver Nanoparticles in Human Peripheral Blood Mononuclear Cells. *Nanomaterials* **2020**, *10*, 1–18, doi:10.3390/nano10071390.

50. Maurer, L.L.; Meyer, J.N. A Systematic Review of Evidence for Silver Nanoparticle-Induced Mitochondrial Toxicity. *Environmental Science: Nano* **2016**, *3*, 311–322, doi:10.1039/c5en00187k.

51. Ahn, J.M.; Eom, H.J.; Yang, X.; Meyer, J.N.; Choi, J. Comparative Toxicity of Silver Nanoparticles on Oxidative Stress and DNA Damage in the Nematode, Caenorhabditis Elegans. *Chemosphere* **2014**, *108*, 343–352, doi:10.1016/j.chemosphere.2014.01.078.

52. Enea, M.; Pereira, E.; de Almeida, M.P.; Araújo, A.M.; Bastos, M. de L.; Carmo, H. Gold Nanoparticles Induce Oxidative Stress and Apoptosis in Human Kidney Cells. *Nanomaterials* **2020**, *10*, 1–17, doi:10.3390/nano10050995.

53. Kalvelytė, A. V.; Imbrasaitė, A.; Krestnikova, N.; Stulpinas, A. Adult Stem Cells and Anticancer Therapy. *Advances in Molecular Toxicology* **2017**, *11*, 123–202, doi:10.1016/B978-0-12-812522-9.00004-X.

54. Park, J.; Lee, J.; Choi, C. Mitochondrial Network Determines Intracellular ROS Dynamics and Sensitivity to Oxidative Stress through Switching Inter-Mitochondrial Messengers. *PLoS ONE* **2011**, *6*, doi:10.1371/journal.pone.0023211.

55. Holmila, R.J.; Vance, S.A.; King, S.B.; Tsang, A.W.; Singh, R.; Furdui, C.M. Silver Nanoparticles Induce Mitochondrial Protein Oxidation in Lung Cells Impacting Cell Cycle and Proliferation. *Antioxidants* **2019**, *8*, doi:10.3390/antiox8110552.

56. Gopinath, P.; Gogoi, S.K.; Sanpui, P.; Paul, A.; Chattopadhyay, A.; Ghosh, S.S. Signaling Gene Cascade in Silver Nanoparticle Induced Apoptosis. *Colloids and Surfaces B: Biointerfaces* **2010**, *77*, 240–245, doi:10.1016/j.colsurfb.2010.01.033.

57. Gurunathan, S.; Park, J.H.; Han, J.W.; Kim, J.H. Comparative Assessment of the Apoptotic Potential of Silver Nanoparticles Synthesized by Bacillus Tequilensis and Calocybe Indica in MDA-MB-231 Human Breast Cancer Cells: Targeting P53 for Anticancer Therapy. *International Journal of Nanomedicine* **2015**, *10*, 4203–4223, doi:10.2147/IJN.S83953.

58. Plackal Adimuriyil George, B.; Kumar, N.; Abrahamse, H.; Ray, S.S. Apoptotic Efficacy of Multifaceted Biosynthesized Silver Nanoparticles on Human Adenocarcinoma Cells. *Scientific Reports* **2018**, *8*, 1–14, doi:10.1038/s41598-018-32480-5.

59. Xu, Z.; Feng, Q.; Wang, M.; Zhao, H.; Lin, Y.; Zhou, S. Green Biosynthesized Silver Nanoparticles With Aqueous Extracts of Ginkgo Biloba Induce Apoptosis via Mitochondrial Pathway in Cervical Cancer Cells. *Frontiers in Oncology* **2020**, *10*, 1–10, doi:10.3389/fonc.2020.575415.

60. Jabeen, S.; Qureshi, R.; Munazir, M.; Maqsood, M.; Munir, M.; Shah, S.S.H.; Rahim, B.Z. Application of Green Synthesized Silver Nanoparticles in Cancer Treatment - A Critical Review. *Materials Research Express* **2021**, *8*, 92001, doi:10.1088/2053-1591/ac1de3.

61. Piao, M.J.; Kang, K.A.; Lee, I.K.; Kim, H.S.; Kim, S.; Choi, J.Y.; Choi, J.; Hyun, J.W. Silver Nanoparticles Induce Oxidative Cell Damage in Human Liver Cells through Inhibition of Reduced Glutathione and Induction of Mitochondria-Involved Apoptosis. *Toxicology Letters* **2011**, *201*, 92–100, doi:10.1016/j.toxlet.2010.12.010.

62. Selck, H.; Handy, R.D.; Fernandes, T.F.; Klaine, S.J.; Petersen, E.J. Nanomaterials in the Aquatic Environment : An EU-USA Perspective on the Status of Ecotoxicity Testing , Research Priorities and Challenges Ahead. *Environmental Toxicology and Chemistry* **2016**, *35*, 1055–1067, doi:10.1002/etc.3385.Nanomaterials.

63. Bajak, E.; Fabbri, M.; Ponti, J.; Gioria, S.; Ojea-jiménez, I.; Collotta, A.; Mariani, V.; Gilliland, D.; Rossi, F.; Gribaldo, L. Changes in Caco-2 Cells Transcriptome pro Fi Les upon Exposure to Gold Nanoparticles. *Toxicology Letters* **2015**, *233*, 187–199, doi:10.1016/j.toxlet.2014.12.008.

64. Wusu, A.D.; Sibuyi, N.R.S.; Moabelo, K.L.; Goboza, M.; Madiehe, A.; Meyer, M. Citrate-Capped Gold Nanoparticles with a Diameter of 14 Nm Alter the Expression of Genes Associated with Stress Response , Cytoprotection and Lipid Metabolism in CaCo-2 Cells. *Nanotechnology* **2022**, *33*, 1–11.

65. Schroeder, B.; Steen, T. Vander; Espinoza, I.; Venkatapoorna, C.M.K.; Hu, Z. Fatty Acid Synthase (FASN) Regulates the Mitochondrial Priming of Cancer Cells. **2021**, 1–10, doi:10.1038/s41419-021-04262-x.

66. Mayas, M.D.; Ortega, F.J.; Macías-González, M.; Bernal, R.; Gómez-huelgas, R.; Fernández-real, J.M.; Tinahones, F.J. Inverse Relation between FASN Expression in Human Adipose Tissue and the Insulin Resistance Level. **2010**, 1–7.

67. Veigel, D.; Wagner, R.; Stubiger, G.; Wuczkowski, M.; Filipits, M.; Horvat, R.; Benham, B.; Lopez-Rodrıguez, M.L.; Leisser, A.; Valent, P.; et al. Fatty Acid Synthase Is a Metabolic Marker of Cell Proliferation Rather than Malignancy in Ovarian Cancer and Its Precursor Cells. *International Journal of Cancer* **2015**, *136*, 2078–2090, doi:10.1002/ijc.29261.

68. Li, L.-Y.; Yang, Q.; Jiang, Y.-Y.; Yang, W.; Jiang, Y.; Li, X.; Hazawa, M.; Zhou, B.; Huang, G.-W.; Xu, X.-E.; et al. Interplay and Cooperation between SREBF1 and Master Transcription Factors Regulate Lipid Metabolism and Tumor-Promoting Pathways in Squamous Cancer. *Nature Communications* **2021**, *12*, 1–17, doi:10.1038/s41467-021-24656-x.

69. Bertolio, R.; Napoletano, F.; Mano, M.; Maurer-stroh, S.; Fantuz, M.; Zannini, A.; Bicciato, S.; Sorrentino, G.; Sal, G. Del Sterol Regulatory Element Binding Protein 1 Couples Mechanical Cues and Lipid Metabolism. *Nature Communications* **2019**, *10*, 1–11, doi:10.1038/s41467-019-09152-7.

70. Wang, M.; Wang, K.; Liao, X.; Hu, H.; Chen, L.; Meng, L.; Gao, W.; Li, Q. Carnitine Palmitoyltransferase System : A New Target for Anti-In Fl Ammatory and Anticancer Therapy ? *Frontiers in Pharmacology* **2021**, *12*, 1–16, doi:10.3389/fphar.2021.760581.

71. Zhang, X.; Zhang, Z.; Liu, S.; Li, J.; Wu, L.; Lv, X.; Xu, J.; Chen, B.; Zhao, S.; Yang, H. CPT2 Down-Regulation Promotes Tumor Growth and Metastasis through Inducing ROS / NF ? B Pathway in Ovarian Cancer. *Translational Oncology* **2021**, *14*, doi:10.1016/j.tranon.2021.101023.

72. Ma, Y.; Temkin, S.M.; Hawkridge, A.M.; Guo, C.; Wang, W.; Wang, X.; Fang, X. Fatty Acid Oxidation: An Emerging Facet of Metabolic Transformation in Cancer. *Cancer letters* **2019**, *435*, 92–100, doi:10.1016/j.canlet.2018.08.006.Fatty.

73. Uxa, S.; Castillo-Binder, P.; Kohler, R.; Stangner, K.; Müller, G.A.; Engeland, K. Ki-67 Gene Expression. *CDDpress* **2021**, *28*, 3357–3370, doi:10.1038/s41418-021-00823-x.

74. Li, P.; Zhou, L.; Zhao, T.; Liu, X.; Zhang, P. Caspase-9 : Structure , Mechanisms and Clinical Application. *Oncotarget* **2017**, *8*, 23996–24008.

75. Brentnall, M.; Rodriguez-menocal, L.; Guevara, R.L. De; Cepero, E.; Boise, L.H. Caspase-9 , Caspase-3 and Caspase-7 Have Distinct Roles during Intrinsic Apoptosis. *BMC Cell Biology* **2013**, *14*, 1–9.

76. Emeny, R.T.; Baumert, J.; Zannas, A.S.; Kunze, S.; Wahl, S.; Iurato, S.; Arloth, J.; Erhardt, A.; Balsevich, G.; Schmidt, M. V; et al. Anxiety Associated Increased CpG Methylation in the Promoter of Asb1 : A Translational Approach Evidenced by Epidemiological and Clinical Studies and a Murine Model. *Neuropsychopharmacology* **2017**, *43*, 342–353, doi:10.1038/npp.2017.102.

77. Maugeri, N.; Radhakrishnan, J.; Knight, J.C. Genetic Determinants of HSP70 Gene Expression Following Heat Shock. *Human Molecular Genetics* **2010**, *19*, 4939–4947, doi:10.1093/hmg/ddq418.

78. Radons, J. The Human HSP70 Family of Chaperones : Where Do We Stand ? *Cell Stress and Chaperones* **2016**, *21*, 379–404, doi:10.1007/s12192-016-0676-6.

79. Leak, R.K. Heat Shock Proteins in Neurodegenerative Disorders and Aging. *Journal of Cell Communication and Signaling* **2014**, *8*, 293–310, doi:10.1007/s12079-014-0243-9.

80. Andersen, V.; Vogel, L.K.; Kopp, T.I.; Sæb, M.; Nonboe, A.W.; Hamfjord, J.; Kure, E.H.; Vogel, U. High ABCC2 and Low ABCG2 Gene Expression Are Early Events in the Colorectal Adenoma- Carcinoma Sequence. *PLoS ONE* **2015**, *10*, 1–13, doi:10.1371/journal.pone.0119255.

81. Chen, Y.; Zhou, H.; Yang, S.; Su, D. Increased ABCC2 Expression Predicts Cisplatin Resistance in Non-Small Cell Lung Cancer. *cell biochemitry and function WILEY* **2021**, *39*, 277–286, doi:10.1002/cbf.3577.

82. Kielar, D.; Kaminski, W.E.; Liebisch, G.; Piehler, A.; Wenzel, J.J.; Christoph, M.; Heimerl, S.; Langmann, T.; Friedrich, S.O.; Alfred, B.; et al. Adenosine Triphosphate Binding Cassette (ABC) Transporters Are Expressed and Regulated During Terminal Keratinocyte Di¡erentiation: A Potential Role for ABCA7 in Epidermal Lipid Reorganization. *THE JOURNAL OF INVESTIGATIVE DERMATOLOGY* **2003**, *121*, 465–474, doi:10.1046/j.1523-1747.2003.12404.x.

83. Badmus, J.A.; Ekpo, O.E.; Sharma, J.R.; Sibuyi, N.R.S.; Meyer, M.; Hussein, A.A.; Hiss, D.C. An Insight into the Mechanism of Holamine- and Funtumine-Induced Cell Death in Cancer Cells. *Molecules* **2020**, *25*, 1–16.

84. Meyer, M.; Essack, M.; Kanyanda, S.; Rees, J.G. A Low-Cost Flow Cytometric Assay for the Detection and Quantification of Apoptosis Using an Anionic Halogenated Fluorescein Dye. *BioTechniques* **2008**, *45*, 317–320, doi:10.2144/000112908.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.1 General discussion

The complexity of chronic wound treatments makes the discovery of wound healing drug formulations a greatly important task. To date, many formulations have been discovered, however because wound healing is a complicated and sensitive process most of these formulations are not fully effective. This means continuous research to find more effective wound healing formulations, is needed. Nanoformulations have been gaining popularity because of their increased and enhanced activity in wound healing (Tyavambiza, Dube, et al., 2021). Many nanoparticles and nanocomposites have been reported to enhance antimicrobial and anti-inflammatory activities at the wound site. They have also been associated with enhanced keratinocytes and fibroblasts proliferation and migration, collagen deposition and angiogenesis (Kalantari et al., 2020). Toxicity is a major problem with many nanoformulations; some have been reported to cause excessive toxicity to normal healthy cells at the wound area in particular fibroblasts and keratinocytes. This disrupts the healing process and reduces the effectiveness of the formulations. To tackle the problem of toxicity, nanomaterials have been synthesized using biological materials such as plants. This synthesis excludes the use of toxic chemicals and therefore produces more biocompatible nanomaterials (Aboyewa et al., 2021).

In this study silver nanoparticles (AgNPs) synthesized using *C. orbiculata* water extract (*Cotyledon*-AgNPs) were investigated for their wound healing activities and their toxicity effects on normal skin fibroblasts. The traditional uses of *C. orbiculata* as a treatment for skin wounds, acne, boils and skin eruptions suggests that it has wound healing properties (Maroyi, 2019). Previously, we investigated the antimicrobial and inflammatory activities of the *Cotyledon*-AgNPs and the results showed that the *Cotyledon*-AgNPs do possess good antimicrobial and anti-inflammatory properties even more than the *C. orbiculata* extract (Tyavambiza, Elbagory, et al., 2021). These findings strongly suggest that the *Cotyledon*-AgNPs might have wound healing properties.

The wound healing activities were tested using the in vitro wound scratch assay. However, before this assay, the cell viability and growth promoting effects of the treatments on KMST-6, HaCaT and CHO cell lines were determined using the WST-1 assay (Goboza et al., 2020). The viability of cells treated with the *C. orbiculata* water extract and the *Cotyledon*-AgNPs was both

dose and time dependant. At lower concentrations the *Cotyledon*-AgNPs increased the rate of cell growth. At concentrations between 0.1 – 2.5 µg/ml, *Cotyledon*-AgNPs increased the growth of KMST-6 and CHO cells at both 24 and 72 h. The *Cotyledon*-AgNPs also increased the growth rate of HaCaT cells but at concentrations between 0.1 – 2.5 and 0.1 – 1.3 µg/ml at 24 and 72 h respectively. The *C. orbiculata* water extract did not reduce the viability of HaCaT and CHO cells at both 24 and 72 h incubation. However, they reduced the viability of KMST-6 cells at concentrations higher than 240 µg/ml. The *C. orbiculata* water extract promoted cell growth at most of the concentrations tested especially the lower concentrations. The concentrations of 2.5 µg/ml (*Cotyledon*-AgNPs) and 15 µg/ml (*C. orbiculata* water extract) were used for the scratch wound healing assay as these showed a stable trend amongst the different cell lines.

Following these results, the wound healing scratch assay was done using concentrations that were not toxic to the cells but promoted cell growth (15 and 2.5 µg/ml). In this assay, cells were scratched off a cellular monolayer in a cell culture plate. The scratched surface is an in vitro representation of a wound (Liang et al., 2007). It was shown that for both *Cotyledon*-AgNPs and *C. orbiculata* extracts treated cells, the scratched gap closes faster than for the untreated control. The cells also increased in number (proliferation) while the scratched gap closed through migration. Results obtained from this assay showed that both *Cotyledon*-AgNPs and *C. orbiculata* extracts possess wound healing activities through increased fibroblast and keratinocytes migration and proliferation. The results also showed that different cells have different proliferation and migration rates. Figure 3 in Chapter 3 showed that HaCaT cells migrated faster than CHO and KMST-6 cells hence the scratched gap closed faster in these cells.

To confirm the wound healing capabilities of the *Cotyledon*-AgNPs and *C. orbiculata* extracts, gene expression studies were done using a panel of wound healing related genes. The expression of 86 genes involved in different processes such as (signal transduction, cell growth, inflammation and extracellular matrix formation) was investigated. In *Cotyledon*-AgNPs treated cells, 17 of these genes were differentially expressed. Genes with a fold change of $\geq \pm 1.5$ and p-values of <0.05 were considered to be differentially expressed. Eight (8) of the differentially

expressed genes (DEGs) were upgregulated while 9 were downregulated. The upregulated genes include CDH1, COL14A1, EGF, FGA, FGF10, ITGB1, ITGB6 and PTGS2. These genes are involved in epithelial cell proliferation and migration (especially in keratinocytes and fibroblasts), increase of tensile strength to new skin, improved collagen construction and formation of the ECM. The upregulated genes are also involved in cellular adhesion which is essential in cell communication and tissue development. The downregulated genes include CCL2, CTGF, CXCL2, FGF2, HBEGF, IL6, ITGA2, MMP2 and SERPINE1. These genes are mainly involved in inflammation. Prolonged inflammation usually leads to chronic wounds therefore anti-inflammatory agents (such as *Cotyledon*-AgNPs) may enhance the wound healing process by reducing prolonged inflammation during wound healing.

The effects of *C. orbiculata* extract on the wound healing related genes, was also investigated. Of the 86 tested genes, only 11 were differentially expressed and all of them were upregulated. These genes include COL5A3, ACTC1, FGF7, WNT5A, ITGB6, TAGLN, ITGB1, VTN, ITGA4, CSF2 and FGF10. They are involved in epithelial cell proliferation and migration, collagen construction, cell adhesion, cytoskeletal organisation, and cell motility (Fathke et al., 2006; Vuga et al., 2009; Elsafadi et al., 2020). These genes support cell differentiation, proliferation and migration ultimately promoting wound healing. It was concluded that both *Cotyledon*-AgNPs and *C. orbiculata* extracts do possess wound healing activities and they use similar pathways and mechanism in promoting wound healing. The gene expression results also confirmed the findings obtained from the cell viability and wound scratch assays. These findings validate the traditional use of *C. orbiculata* in wound treatment, It is also the first scientific study to show the mechanism in which the *C. orbiculata* promotes wounds healing.

The presence of phytochemicals and the antioxidant activities of the *Cotyledon*-AgNPs and *C. orbiculata* extracts were also investigated. It was shown that they both contain polyphenols including flavanols, flavonols and tanins. They were also shown to have good antioxidant activities as they showed good reducing capabilities in the FRAP and ABTS assays. The present phytochemicals maybe the ones responsible for the biological activities exerted by the *Cotyledon*-AgNPs and the *C. orbiculata* extract. After exhibiting good antioxidant and wound healing activity, it was imperative to determine the toxic effects of the *Cotyledon*-AgNPs and *C. orbiculata* extracts towards healthy cells that can be found at the wound site. Prolonged use of

high doses of silver has been reported to cause agyria, a skin discoloration condition. Some silver formulations were reported to be toxic to keratinocytes and fibroblast while some exhibited systematic cytotoxicity (Khansa et al., 2019). Nanoparticles exert toxicity through ROS generation, mitochondrial dysfunction, membrane damage or apoptosis. In this study the cytotoxicity effects of *Cotyledon*-AgNPs and the *C. orbiculata* extract in KMST-6 cells were determined using bioassays which include ROS production, mitochondrial membrane potential and APOPercentage.

Firstly, the IC_{50} values of the *Cotyledon*-AgNPs and *C. orbiculata* extract were determined. It was shown that the *C. orbiculata* extract had higher IC_{50} values compared to the *Cotyledon*-AgNPs and is therefore less toxic than the *Cotyledon*-AgNPs synthesized from it. The results also showed that the *Cotyledon*-AgNPs were toxic to the different cells at different concentrations; however these were much higher than the concentrations at which the *Cotyledon*-AgNPs and *C. orbiculata* extract exert their wound healing properties. Cellular uptake studies to confirm the uptake of *Cotyledon*-AgNPs by the cells were done. This was achieved using dark field microscopy which showed that the *Cotyledon*-AgNPs were successfully taken up in the cytoplasm and on the membrane of the KMST-6 cells.

Following successful uptake, nanoparticles in the cytoplasm or nucleus can increase ROS levels leading to toxicity. Excessive amounts of ROS can disrupt the cellular antioxidant defence system therefore damaging the cells (Chairuangkitti et al., 2013). The effects of *Cotyledon*-AgNPs and *C. orbiculata* extract on ROS levels in keratinocytes, fibroblast and epithelial cells were investigated. Both the *Cotyledon*-AgNPs and the *C. orbiculata* extract did not induce any significant increase in ROS levels in all the tested cell lines. The effects of the *Cotyledon*-AgNPs and the *C. orbiculata* extracts on MMP were investigated using the TMRE assay. It was shown that cells treated with the *Cotyledon*-AgNPs or *C. orbiculata* extract retained the TMRE dye, indicating that the mitochondria were active in these cells and that there was no significant mitochondrial membrane depolarization. Both treatments showed a similar response to that of the untreated control. These results supported the findings obtained in the oxidative stress test, as mitochondrial membrane depolarization would have led to increased ROS levels and vice versa. The APOPercentage assay was used to determine the effects of *Cotyledon*-AgNPs and *C. orbiculata* extract on apoptosis in KMST-6 cells. Both treatments did not have significant

apoptotic effects on the cells as the recorded levels of apoptosis in the treated cells were not significantly higher than that obtained in the untreated cells.

To confirm the results obtained from the 3 assays, gene expression studies were done. A human molecular toxicology gene panel consisting of 86 genes was used for the analysis. Only 8 (FASN, SREBF1, CPT2, ASB1, HSPA1B, ABCC2, CASP9 and MKI67) of these genes were differentially expressed. The upregulated genes are involved in steatosis, β-oxidation, cholestasis, apoptosis, and mitochondrial energy metabolism; they also support cell growth and nourishment. Using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) software, the genes were clustered into functional network groups. The upregulated genes clustered into 2 groups while some of the genes did not form part of any known functional network. The clustered groups consisted of 3 (FASN, CPT2 and SREBF1) and 2 (MKI-67 and CASP9) genes while 3 (ASB1, HSPA1B and ABCC2) other genes did not associate into clusters. FASN, CPT2 and SREBF1 are involved in lipid metabolism, oxidation and haemostasis, respectively. When controlled lipid metabolism promotes cell nutrition and growth.

The observed upregulation of the gene MKI-67 in cluster B correlates with the upregulation of the gene, FASN. MKI-67 is a proliferation marker, expressed in proliferating cells and absent in resting cells. Increased expression of MKI-67 has been used as an indicator for cell proliferation (Uxa et al., 2021). Interestingly in this cluster, CASP9, a gene with effects opposite to the MKI-67 was also upregulated. CASP9 is an apoptotic gene which is involved in the activation of caspases responsible for apoptosis. The upregulation of the CASP9 was probably the counteraction by the cells as they were trying to return to haemostasis after increased cellular proliferation. Even though CASP9 was upregulated, it did not induce apoptosis of the *Cotyledon*-AgNPs treated fibroblasts as shown by the APOPercentage assay.

The 3 genes that did not form part of any functional network are ASB1, HSPA1B and ABCC2. ASB1 is involved in the process of ubiquitination which includes protein modification or misfolding which occur because of cell damage. It is also associated with the expression of proinflammatory genes (Emeny et al., 2017). The effects of ASB1 might not be expressed because of the upregulation of the HSPA1B gene. The HSPA1B gene encodes a member of

heat shock proteins found in the cytoplasm. This group of genes protect cells from a range of stresses including proteotoxic stress by identifying and repairing misfolded or damaged proteins and preventing the aggregation of modified proteins (Maugeri et al., 2010; Radons, 2016). As part of the ABC transport proteins, ABCC2 transports various compounds including fatty acids and lipid compounds across the cell membranes and epithelial barriers (Chen et al., 2021). ABCC2 might have been upregulated to transport fatty acids and lipids produced because of the upregulated FASN and SERBF1 genes. The gene panel used in this study investigated the expression of genes involved in oxidative stress, DNA damage and necrosis. The study shows that the expression of these genes was not affected by treatment with Cotyledon-AgNPs. This is in agreement with the findings of the various bioassays (ROS, TMRE and APOPercentage) which demonstrated that the nanoparticles are not toxic. In fact, the gene expression study support previous findings (Chapter 3) which suggest that Cotyledon-AgNPs may promote cell growth as shown by the upregulation of FASN, SERBF1, MKI-67 and HSPA1B.

5.2 Conclusion

From this study, it can be concluded that the medicinal plant, *C. orbiculata* and the silver nanoparticles synthesized from its water extract do possess antioxidant and wound healing activities and are not toxic to normal keratinocytes, fibroblasts and epithelial cells. Although they both showed good activity, the *Cotyledon*-AgNPs were more active than the *C. orbiculata* extract. This can be attributed to the small size of nanoparticles which increase their surface area to volume ratio, hence increasing their activity. Phytochemicals present on the nanoparticles may also play a role in increasing their bioactivity. The *C. orbiculata* water extract and the nanoparticles promote wound healing through keratinocyte and fibroblast proliferation and migration, collagen construction, ECM formation and suppressing excessive inflammation. It was also promising to note that both treatments were not cytotoxic at the highly active concentrations. The molecular toxicity gene panel actually supported the proliferative and anti-inflammatory activities of the nanoparticles. The *C. orbiculata* water extracts and the *Cotyledon*-AgNPs can be used in discovering effective, affordable and safer compounds and nano-formulations that can be useful in wound healing. As this is a plant found in South Africa, discovered formulations will be a great help in fighting chronic wounds in developing countries. It is however recommended to further assess the toxicity of *Cotyledon*-AgNPs on the environment specifically on entities where the nanoparticles might end up after use such as

water bodies and soil. For future studies, wound healing activities of *C. orbiculata* water extracts and the *Cotyledon*-AgNPs can be tested on animal models.

References

Aboyewa, J.A., Sibuyi, N.R.S., Meyer, M. & Oguntibeju, O.O. 2021. Green Synthesis of Metallic Nanoparticles Using Some Selected Medicinal Plants from Southern Africa and Their Biological Applications. *Plants*, 10(9): 1929. https://doi.org/10.3390/plants10091929

Chairuangkitti, P., Lawanprasert, S., Roytrakul, S., Aueviriyavit, S., Phummiratch, D., Kulthong, K., Chanvorachote, P. & Maniratanachote, R. 2013. Silver nanoparticles induce toxicity in A549 cells via ROS-dependent and ROS-independent pathways. *Toxicology in vitro : an international journal published in association with BIBRA*, 27(1): 330–338. https://doi.org/10.1016/j.tiv.2012.08.021

Chen, Y., Zhou, H., Yang, S. & Su, D. 2021. Increased ABCC2 expression predicts cisplatin resistance in non-small cell lung cancer. *cell biochemitry and function WILEY*, 39(2): 277–286. https://doi.org/10.1002/cbf.3577

Elsafadi, M., Manikandan, M., Almalki, S., Mahmood, A., Shinwari, T., Vishnubalaji, R., Mobarak, M., Alfayez, M., Aldahmash, A., Kassem, M. & Alajez, N.M. 2020. Transgelin is a poor prognostic factor associated with advanced colorectal cancer (CRC) stage promoting tumor growth and migration in a TGFβ-dependent manner. *Cell Death & Disease 2020 11:5*, 11(5): 1–13. https://doi.org/10.1038/s41419-020-2529-6

Emeny, R.T., Baumert, J., Zannas, A.S., Kunze, S., Wahl, S., Iurato, S., Arloth, J., Erhardt, A., Balsevich, G., Schmidt, M. V, Weber, P., Kretschmer, A., Pfeiffer, L., Kruse, J., Strauch, K., Roden, M., Herder, C., Koenig, W., Gieger, C., Waldenberger, M., Peters, A., Binder, E.B. & Ladwig, K.-H. 2017. Anxiety Associated Increased CpG Methylation in the Promoter of Asb1 : A Translational Approach Evidenced by Epidemiological and Clinical Studies and a Murine Model. *Neuropsychopharmacology*, 43(2): 342–353.

Fathke, C., Wilson, L., Shah, K., Kim, B., Hocking, A., Moon, R. & Isik, F. 2006. Wnt signaling induces epithelial differentiation during cutaneous wound healing. *BMC Cell Biology*, 7(1): 1–9. doi:10.1186/1471-2121-7-4

Goboza, M.; Meyer, M.; Aboua, Y.G.; Oguntibeju, O.O. 2020. Molecules In Vitro Antidiabetic and Antioxidant Effects of Different Extracts of Catharanthus Roseus and Its Indole Alkaloid, Vindoline. *Molecules*. *25*: 5546. https://doi.org/10.3390/molecules25235546.

Kalantari, K., Mostafavi, E., Afifi, A.M., Izadiyan, Z., Jahangirian, H., Rafiee-Moghaddam, R. & Webster, T.J. 2020. Wound Dressings Functionalized with Silver Nanoparticles: Promises and Pitfalls. *Nanoscale*, 12: 2268–2291. DOI: 10.1039/C9NR08234D

Khansa, I., Schoenbrunner, A.R., Kraft, C.T. & Janis, J.E. 2019. Silver in Wound Care—Friend or Foe?:A Comprehensive Review. *Plastic and Reconsructive Surgery Global Open*, 7(8): 1–10. DOI: 10.1097/GOX.0000000000002390

Liang, C.-C., Park, A.Y. & Guan, J.-L. 2007. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature Protocols 2007 2:2*, 2(2): 329–333. doi:10.1038/nprot.2007.30

Maroyi, A. 2019. A review of botany, medicinal uses, phytochemistry and biological activities of Cotyledon orbiculata. *Journal of Pharmaceutical Sciences and Research*, 11(10): 3491–3496.

Maugeri, N., Radhakrishnan, J. & Knight, J.C. 2010. Genetic determinants of HSP70 gene expression following heat shock. *Human Molecular Genetics*, 19(24): 4939–4947. doi:10.1093/hmg/ddq418

Radons, J. 2016. The human HSP70 family of chaperones : where do we stand ? *Cell Stress and Chaperones*, 21: 379–404. DOI 10.1007/s12192-016-0676-6

Tyavambiza, C., Dube, P., Goboza, M., Meyer, S., Madiehe, A.M. & Meyer, M. 2021. Wound Healing Activities and Potential of Selected African Medicinal Plants and Their Synthesized Biogenic Nanoparticles. *Plants.* 10(12): 2635. https://doi.org/10.3390/plants10122635

Tyavambiza, C., Elbagory, A.M., Madiehe, A.M., Meyer, M. & Meyer, S. 2021. The Antimicrobial and Anti-Inflammatory Effects of Silver Nanoparticles Synthesised from Cotyledon orbiculata Aqueous Extract. *Nanomaterials*. 11(5): 1343. https://doi.org/10.3390/nano11051343

Uxa, S., Castillo-Binder, P., Kohler, R., Stangner, K., Müller, G.A. & Engeland, K. 2021. Ki-67 gene expression. *CDDpress*, 28: 3357–3370. https://doi.org/10.1038/s41418-021-00823-x

Vuga, L.J., Ben-Yehudah, A., Kovkarova-Naumovski, E., Oriss, T., Gibson, K.F., Feghali-Bostwick, C. & Kaminski, N. 2009. WNT5A Is a Regulator of Fibroblast Proliferation and Resistance to Apoptosis. *American Journal of Respiratory Cell and Molecular Biology*, 41(5): 583. DOI: 10.1165/rcmb.2008-0201OC