The wound healing effect of exosomes derived from *Lobostemon fruticosus*

by

Yashmika Rajcoomar

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Supervisors:

Dr. Khonzisizwe Somandi, Dr. Mduduzi N. Sithole, Mr. Lefa Lerotholi and Prof. Yahya E. Choonara

Wits Advanced Drug Delivery Platform Research Unit, Department of Pharmacy and Pharmacology, School of Therapeutic Science, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, 7 York Road, Parktown, 2193, South Africa

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Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.

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31st day of May 2024

Abstract

Exosomes are increasingly being researched and recognized as a novel mode of intercellular communication which can potentially play a significant role in many cellular processes, including immune responses, signal transductions and antigen presentation. Exosomes are membrane bound nanovesicles produced by both mammalian and plant cells. Developing research is mainly focused on their ability to act as a drug delivery vehicle. Other research interests around exosomes are their therapeutic effects for many common diseases including cancer and chronic inflammation. Plant-derived exosomes have emerged as potential candidates for many clinical and therapeutic applications.

The aim of this project was to isolate exosomes derived from *Lobostemon fruticosus* leaves and to investigate the wound healing potential from this plant species.

The plant-derived exosomes were isolated by ultracentrifugation and were characterised by means of scanning electron microscopy (SEM), ZetaSizer, Energy-dispersive Xray spectroscopy and the Pierce™ BCA Protein Assay Kit. The wound healing potential was assessed by I*n vitro* scratch assay using human keratinocytes (HaCaT).

The exosomes displayed a round, spherical shape and had diameters ranging from 41 to 67 nm – falling within range of nano-sized exosomes. The exosomes had a mean particle size of 166.2 d.nm. Chemical analysis of the samples using energy dispersive spectroscopy revealed the presence of carbon, oxygen, potassium, chloride, gold, palladium and sodium. The protein quantification results revealed the exosomes were rich in proteins. The cell viability results, using various concentrations of *Lobostemon fruticosus* exosomes, revealed non-cytotoxicity on HaCaT cells. The *In vitro* scratch assay demonstrated that the exosomes enhanced the migration ability of HaCaT cells in a time dependent manner. The findings suggest and reveal that exosomes derived from *Lobostemon fruticosus* could accelerate the healing process and can be employed as a future drug delivery platform. Further research is required to establish the exact mechanism of action of the healing potential of this plant specie's constituents. Overall results suggest that exosomes derived from *Lobostemon fruticosus* are promising as a potential agent for skin regeneration.

Plagiarism declaration

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Dedication

“Life has a way of testing a person’s will, either by having nothing happen at all or by having everything happen at once.”

- Paulo Coelho

Mom & dad, my greatest strength and biggest weakness, and to everyone who has supported me throughout this journey. Thank you for helping me see this adventure through to the end.

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Abbreviations and Nomenclature

5-FU	5-Fluorouracil
%	Percent
°C	Degrees Celsius
µM	Micrometre
µg	Microgram
AMPK	Adenosine monophosphate activated protein kinase
BCA	Bicinchoninic acid
CBD	Cannabidiol
CircRNAs	Circular RNAs
CO^2	Carbon Dioxide
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
ESCRT	Endosomal sorting complex needed for transport
EVs	Extracellular vesicles
FBS	Fetal bovine serum
g/ml	Grams per millilitre
HaCaT	Cultured human keratinocytes
H.C-EVs	High-Cannabidiol extracellular vesicles
HDF	Human dermal fibroblasts
HIV	Human immunodeficiency virus
HUVEC	Human umbilical vein endothelial cells
H_2O_2	Hydrogen Peroxide
IBS	Irritable bowel syndrome
LncRNAs	Long non-coding RNAs
L.C-EVs	Low-Cannabidiol extracellular vesicles
mRNA	Messenger RNAs
miRNAs	Micro RNAs
mg	Milligram
ml	Millilitre
MISEV	Minimum information for studies of extracellular vesicles
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
P/S	Penicillin–streptomycin
PBS	Phosphate-buffered saline
PLGA	Poly-(d,l-lactic acid-co-glycolic acid)
PDI	Polydispersity index

rAd5	Serotype 5 recombinant adenovirus vectors
ROS	Reactive oxygen species
RNA	Ribonucleic acid
rRNAs	Ribosomal RNAs
SEM	Scanning electron microscopy
(si)RNA	Short interfering RNA
TRAIL	TNF-related apoptosis inducing ligand
TNF	Tumour necrosis factor
USA	United States of America
w/w	Weight per weight

Chapter 1: Background, Aim and Objectives

1.1 Aim

The aim of this project was to investigate whether *Lobostemon fruticosus* exhibited a wound healing potential.

1.2 Objectives

The objectives of this project were to:

- Isolate exosomes derived from *Lobostemon fruticosus* leaves.
- Investigate the wound healing potential from this plant species. This potential was assessed by *In vitro* scratch assay using human keratinocytes (HaCaT).
- Perform characterization tests, namely scanning electron microscopy (SEM), ZetaSizer, Energy-dispersive Xray spectroscopy and the Pierce™ BCA Protein Assay Kit to assess whether the exosomes derived displayed traits of nanoparticles.
- Assess the cell viability of using various concentrations of *Lobostemon fruticosus* exosomes on HaCaT cells.

1.3 Problem statement

With the progression of scientific research and modern medicine, there needs to be treatment alternatives to the current allopathic medicine trends. Although beneficial, allopathic medicine is also known to be harmful and to cause side-effects in patients. Plant-derived exosomes are gaining popularity for being known as the 'animal-free ingredient' in various fields. *Lobostemon fruticosus,* a plant species local to South Africa, has been traditionally used as a medicinal plant for decades. It is said that *Lobostemon fruticosus* exhibits a wide range of therapeutic benefits, one of which is wound healing. Locals claim that this plant can heal a condition in a mere eight days. Given this statement, coupled with the fact that plant-derived exosomes offer a wide range of therapeutic benefits, can exosomes derived from *Lobostemon fruticosus* be used medicinally to accelerate wound healing?

1.4 Hypothesis

If this plant species can show to accelerate the wound healing process using the *In vitro* scratch assay technique, then it can be said that *Lobostemon fruticosus* does indeed exhibit wound healing potential.

Chapter 2: Literature Review

2.1 Introduction

Richard Feynman developed the idea for nanotechnology and nanoscience in 1959 [1]. The physicist described a method through which researchers could be able to modify and exert control over individual atoms and molecules. The publication of E. Drexler's book, "*Vehicles of creation: the arrival of the nanotechnology era"*, in 1986 marked the beginning of the nanotechnology era [2].

Nanotechnology involves the creation of minute and more easily absorbed particles on a molecular level [3]. It has a significant role in advanced medicine formulations, targeting site specific areas. Nanotechnology can also be used in delivering a drug to a desired target site in a controlled manner without causing additional adverse effects, and it can further be used in controlling drug release and ensuring successful drug delivery [4]. The National Nanotechnology Initiative in the United States and the American National Science Foundation both state that nanomaterials typically vary from 1 to 100 nm in size [4]. At this minuscule scale, nanoparticles with bigger surface areas per unit volume have higher surface activities, which accelerates chemical reactions and catalysis. Consequently, many processes become more efficient [5]. Nanotechnology endeavours to manipulate matter at the atomic and molecular scales [5]. In recent years, nanomedicine has proven highly beneficial in the treatment of various diseases, serving as anti-cancer agents for chemotherapy [6], biological agents [7], and immunotherapeutic agents [8]. Approximately 80 nano-medicinal products have been approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Due to the advantages that would accrue to the healthcare system, emphasis has been placed on the development of nanomedicines since 2010. This has resulted in the number of marketed nanomedicines significantly increasing. The EMA and the FDA continue to examine the approval of nanoparticles as they are currently the subject of extensive human research [9]. An example of an FDA-approved nanoparticle is Sublocade® (buprenorphine), a monthly injectable formulation comprising of poly-(d,l-lactic acid-co-glycolic acid) (PLGA), which has been available since 2017 for treating opioid dependence in patients. Currently, its safety in patients with sickle cell disease is under investigation [10]. More recently in 2020, the FDA approved the Pfizer pharmaceuticals mRNA vaccine, Pfizer-BioNTech Vaccine, for the protection against the Covid-19 infection [11].

Nanomedicine and nano-based drug delivery systems have garnered significant attention due to their diminutive dimensions and expansive surface area [5]. The minute scale affords enhanced intracellular drug uptake into specific cellular targets and precise bio-distribution. The substantial surface area facilitates facile manipulation of drug-loaded particles with

heightened capacity. Notably, nanoparticles exhibit exceptional structural stability, ensuring sustained drug delivery without degradation—an invaluable attribute. Furthermore, empirical findings demonstrate that nano-carriers possess low cytotoxicity and demonstrate effective biocompatibility [5]. As an illustrative case, Shekh and co-workers (2023) investigated the viability of delivering 5-Fluorouracil (5-FU) to human colon cancer HCT116 cells using an autologous exosome-mediated drug delivery platform. Their findings demonstrated that autologous exosomes loaded with 5-FU exhibited an accelerated drug release rate in acidic environments. The outcomes of cell viability assays indicated an intensified cytotoxic impact on HCT116 cells, indicative of the proficient conveyance of 5-FU by the exosomes [12]. Furthermore, research conducted by Sabzichi *et al*. (2016) showcased that nanostructured lipid carriers loaded with melatonin enhanced cytotoxicity and apoptosis induced by melatonin in MCF-7 breast cancer cells [13]. Consequently, in this review, our primary focus will be on mammalian- and plant-derived extracellular vesicles, which we will refer to as 'plant-derived exosomes,' as the nanoparticles of interest under discussion. The biogenesis, biological composition, therapeutic potential and stability of mammalian- and plant-derived exosomes will be discussed in greater detail.

2.2 Exosomes

Exosomes, which are naturally produced by nearly all eukaryotic cells, are minute extracellular vesicles (EVs) characterized by their diminutive size, typically ranging from 30 to 150 nanometres in diameter, with a density ranging between 1.13 to 1.19 grams per millilitre [14]. However, by 2007, Valadi *et al.* discovered that exosomes contained microRNA (miRNA) that could be transferred between cells [15]. This sparked interest in their potential as both biomarkers, and as therapeutic agents in various diseases including cardiovascular diseases [16]. Exosomes are a subtype of EVs created through the endosomal pathway [17]. Due to their nano-size, biocompatibility and excellent stability, they have drawn a lot of interest as prospective nano-drug delivery systems [17]. Exosomes have a reduced immunogenicity and can cross the blood–brain barrier, which is essential in treating neurologically related diseases [18]. Exosome research has become increasingly popular recently as a result of their potential to aid in the detection and treatment of cancer, as well as neurological and immunological disorders [19]. Further research has revealed that exosomal components are essential regulators of mammalian reproduction and conditions associated with pregnancy as well as being involved in a variety of physiological activities, including reproduction [20].

Exosomes are currently known to have an involvement in gene regulation and the transfer of biologically active substances during cell-to-cell communication [19]. They serve as cargo

proteins that can transport various biomolecules, including proteins, lipids, and nucleic acids, to target cells where they elicit various biological effects [17]. Research has shown that this cargo, which can affect both nearby and distant recipient cells, by attaching to the cell membrane and releasing its contents, indicates the health or sickness status of the source or parent cells. The recipient cells undergo physiological, phenotypical, and functional changes as a result of the release of this intracellular load [14]. These results add to countless studies that have revealed exosomes' potential as new biomarkers and therapeutic agents. This shows how exosomes, as cell-to-cell mediators, are connected to both pathological and physiological processes and have value in a variety of cellular functions, such as immune response, signal transduction, and antigen presentation [14, 19]. In a study conducted by Showalter *et al*. (2019), it was reported that numerous metabolites that are directly linked to immunomodulation are found in exosomes derived from mesenchymal stem cells, including the activation of regulatory T lymphocytes and M2 macrophage polarization [21]. Both plant and mammalian cells produce exosomes [22]. Depending on their source of origin, exosomes have the lipid bilayer which encloses a unique blend of biologically active substances (or ‘cargo’) including metabolites, proteins, lipids and nucleic acids (miRNA and RNA) [14, 22]. The configuration of these components, which serve as a marker for their cell origin, can change the pharmacokinetic properties of the exosomes [23].

Exosomes from mammalian cells are highly acknowledged for their use as disease biomarkers [24, 25]. They also are involved in several pathological and physiological reactions [26]. Valadi *et al.* (2007) found that the biological processes of other cells can be regulated by nucleic acid components found in the mouse mast cell exosomes [27]. Other studies have revealed that exosomes can be transferred to particular organs and tissues via bodily fluids and play a significant role in the regulation of physiological processes. Circular RNAs (circRNAs), long non-coding RNAs (IncRNAs), and miRNAs are all present in exosomes [28]. Exosomes can be secreted by nearly all mammalian cells including mesenchymal cells, neuronal cells, macrophages, mast cells and epithelial cells [23]. Exosomes, which immune cells can produce and which have a substantial impact on the growth and activity of receptor cells, are important in the immunological response to cancer [29]. Body fluids such as urine, serum, saliva, breast milk and cerebrospinal fluid have all been reported to contain exosomes [25]. Exosomes are said to play an integral role in human development with respect to immunity and even neoplasia. For example, as a result of the simple, non-invasive and stable properties of urinary exosomes, urine can contain disease biomarkers as opposed to blood or tissue samples. Urinary exosomes therefore have tremendous potential for prognosis, early screening, monitoring disease progression and treatment of diseases [25].

Studies on exosomes have become much more prevalent recently as a result of their possible role in the detection and treatment of cancer [30], neurodegenerative diseases [14], and immunological disorders [31]. Studies indicate that exosomes are present in a variety of pathological conditions including tumorigenesis, the progression of neurodegenerative diseases and immunological disorders [23].

Exosomes derived from plants have not been extensively studied compared to those originating from mammals, which have been well studied [14]. However, plant-derived exosomes are receiving a lot of attention as they can be obtained from dietary sources. This is motivated by the notion that eating particular foods may have positive health benefits and lower the risk of contracting certain diseases [32]. Since the gastrointestinal tract is the first organ to acquire EVs (i.e., exosomes) from edible fruits and vegetables, many studies have focused on how EVs' biological properties affect the intestinal barrier [33]. By limiting detrimental factors and encouraging healing factors, these studies have demonstrated that EVs can be used to aid in the prevention and treatment of inflammatory bowel syndrome (IBS) [33]. A study by Mu *et al*. (2014) revealed that treatment in mice with grape exosome-like nanoparticles for IBS, prevented the progression of the disease and reduced mortality of the mice [34]. Additionally, the team characterized exosomes from carrot, grapefruit and ginger [34]. Results showed that the anti-inflammatory cytokine IL-10 and the anti-oxidation gene, heme oxygenase-1 (HO-1) were expressed in response to ginger exosomes. Grapefruit, ginger, and carrot exosomes induced nuclear factor (erythroid-derived 2)-like 2 activation [34]. The bioactive components of plant exosomes allow them to impart many therapeutic properties including anti-tumorigenic [35], anti-oxidative [36], anti-cancer [30] and anti-inflammatory properties [37], as well as tissue regeneration [38] and hepatoprotective effects [39]. The possibilities of using exosomes derived from plants as a drug delivery mechanism is emerging and becoming more apparent [22]. Due to their natural medicinal characteristics that can be used for human health, plant-derived nanoparticles are receiving a lot of research attention [22]. Exosomes derived from plants have the potential to deliver medicines effectively in addition to providing nutritional benefits [24]. Being of natural origin, reduced cytotoxicity, enhanced biocompatibility, and the ability to be genetically modified and loaded with various exogenous agents, make the plant exosomes ideal carriers for delivering the therapeutic agents to specific sites [32]. The identification and development of plant-derived exosome nano-carrier systems are hence the subject of extensive investigation [33, 25]. Growing evidence points to the potential use of plant-derived exosomes as biologically active substances in a variety of industries, including food, biomedicine, and pharmaceuticals [25, 24, 32].

Exosomes may therefore be able to provide prognostic data for a variety of illnesses, including chronic inflammation [40], cardiovascular and renal disease [23], infectious disease [10], neurodegenerative disorders [41], lipid metabolic diseases [42] and tumours [43]. These characteristics support the exosomes' vast potential for clinical use in the detection and treatment of illnesses. Exosomes have been shown in numerous studies to be superior to traditional synthetic carriers as a means of drug administration and as circulating biomarkers for the early identification of disease. Optical platforms [44], chemotherapy [45], gene therapy [45], immunotherapy [46], electrochemical and microfluidic platforms [44] are a few examples of recent developments in exosome-based nanotechnology for precision theranostic platforms. Exosome-based nanotechnology advancements therefore increase the therapeutic potency of illness treatment [47].

2.2.1 Biogenesis of exosomes

Though their biogenesis is still debatable, exosomes are frequently produced in the multivesicular endosome compartments of the cell and released upon the fusion of these compartments with the plasma membrane [14]. Exosomes are produced from the endocytic pathway during late endocytosis when membrane invagination leads to the formation of intraluminal vesicles within multivesicular structures [22, 48]. The early endosomes combine with the surrounding membrane layer to form the intraluminal vesicles. Exosomes are enclosed within the multivesicular bodies, which later fuse with plasma membrane and release the contents into the extracellular space. As the exosomes' contents are packed using a strictly controlled procedure, the proteins frequently have post-translational changes like ubiquitination [22].

2.2.1.1 Biogenesis of mammalian-derived exosomes

Exosomes are extensively synthesized by mammalian cells’ double protoplasmic membrane invagination, followed by the inward budding of the luminal membrane of intracellular multivesicular structures. These structures contain intraluminal vesicles [22, 23, 48, 49]. The initial invagination of the plasma membrane results in the formation of early sorting endosomes and include cellular proteins, metabolites, small molecules, lipids and ions [23]. During the second invagination, early sorted endosomes develop into late sorted endosomes, resulting in the formation of multivesicular bodies which contain intraluminal vesicles. Exosome formation is fundamentally regulated by two mechanisms: the ESCRT-dependent and the ESCRT-independent mechanisms [23]. The sorting and transportation of the active ingredients into the intraluminal vesicles is greatly aided by the endosomal sorting complex needed for transport (ESCRT) [50, 51]. The four complexes (ESCRT 0, -I, -II, -III) that make up the endosomal sorting complex necessary for transport are made up of several proteins. By

attaching to and gathering the ubiquitinylated proteins on the cell membrane's surface, ESCRT-0 is essential in isolating them [49]. Signal Transducing Adaptor Molecule and Hepatocyte growth-factor regulated tyrosine kinase substrate, which can connect with the ubiquitinated cargo, are the two subunits that make up ESCRT-0. Additionally, it transports the complex to the site via interacting with the ESCRT-1 protein TSG101. The heterotetrametric complex ESCRT-1 is in charge of categorizing the active ingredients into the multivesicular bodies. Through contact with the ESCRT-II complex, a protein concentration results [50, 51]. The proteins EAP30, EAP45 and EAP 20 make up the hetero-tetramer ESCRT-II. The release of exosomes is triggered by the most important functional component, ESCRT-III, an oligomer of charged multivesicular body protein [49, 50, 51]. It causes the formation of membrane-binding spirals, which results in neck constriction and vesicle buds releasing from the membrane, along with VpS4p/SKD1, a related ATPase. The exosomes are released with the aid of this ATPase's energy [49]. The vesicles are then released after interacting with a particular set of N-ethylmaleimide sensitive factor attachment protein receptors [22].

2.2.1.2 Biogenesis of plant-derived exosomes

In contrast to mammalian cells, the endocytic process is less well understood in plant cells [14]. The Trans Golgi Network, a component of which develops into multivesicular bodies, receives the proteins at the plasma membrane via invagination, budding and through the production of transport vesicles. The proteins, now early endosomes, are directed to the Trans Golgi Network [22, 31]. The majority of the ESCRT complexes involved in the maturation and release of intraluminal vesicles of multivesicular bodies are also largely conserved in plants and carry out similar tasks [52]. ESCRT-I is incorporated into multivesicular bodies as a result of FREE-1, a particular ESCRT component in plants, which localizes to the multivesicular bodies and interacts with it. Its capacity to bind ubiquitin also suggests that this protein has other roles in the production of plant exosomes. The plant ESCRT protein complex activity has a significant role in controlling exosome synthesis during the maturation and sorting of multivesicular bodies, with a few minor exceptions [22]. Despite resembling autophagosomes, they merge with any lytic compartments or adhere to the endocytic pathway [31]. Single membraned vesicles are discharged into the cell wall when they bind with the plasma membrane. Formed by exocyst positive organelles, these vesicles are classified as exosomes [22]. **Figure 2.1** illustrates the biogenesis of plant and mammalian derived exosomes.

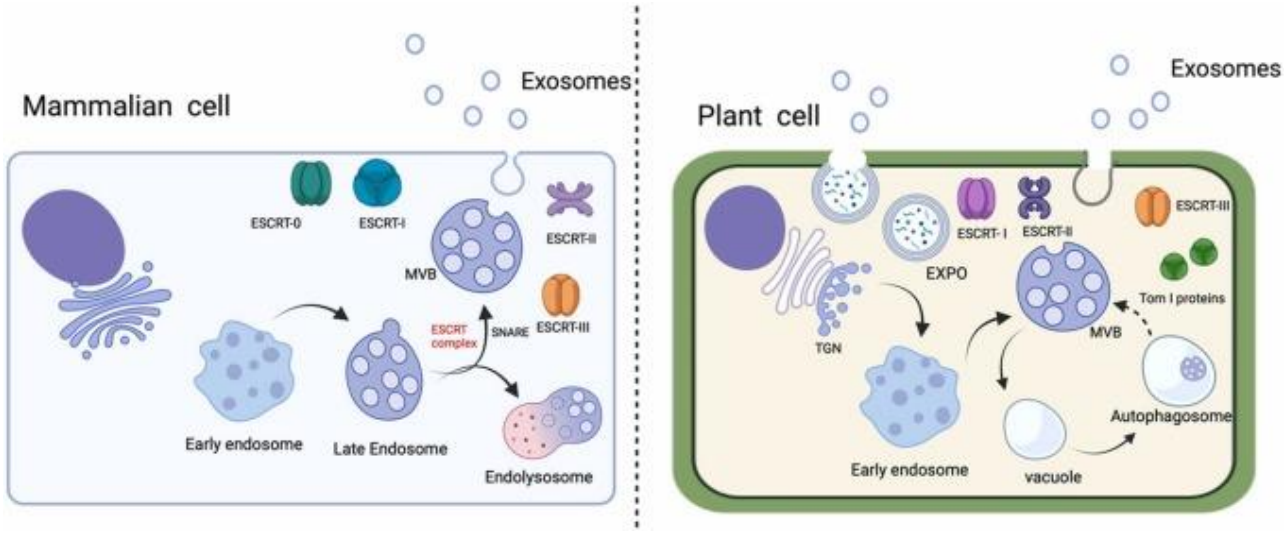


Figure 2.1: *The biogenesis of plant and mammalian derived exosomes* [22].

2.2.2 Biological composition

2.2.2.1 Biological composition of mammalian exosomes

The biological makeup of mammalian exosomes is complex since various species produce different exosomes [23]. Exosomes primarily consist of lipids, proteins and nucleic acids [14, 31].

a) Proteins

The 'marker' tetra-transmembrane proteins CD81, CD63, CD9, and CD82 are found in exosomes [53]. Additionally, endonuclear somatic sorting complex proteins (ALIX and TSG101), heat shock proteins (HSP70 and HSP90), and specific proteins involved in the generation and release of extracellular vesicles (RAB27A, RAB11B, and ARF6) are all concentrated in exosomes [54]. Mammalian exosomes are also rich in transmembrane proteins including EGFR, MHC I, MHC II and glycoproteins [55].

b) Lipids

Various lipids are abundant in exosomes including sphingolipids, gangliosides, unsaturated lipids, phosphatidylcholines cholesterol, diglycerides, glycerophospholipids, phospholipids, and sphingolipids or glycosylceramides [14, 31].

c) Nucleic acids

In addition to messenger RNAs (mRNAs) and miRNAs, other short RNAs have been identified, including ribosomal 18 S and 28 S subunit ribosomal RNAs (rRNAs), specific transfer RNAs

(tRNAs), and other non-coding RNAs like miRNAs and lncRNAs. [31, 56]. In a study, it was discovered exosomes secreted by human mammary cell lines include rRNA as one of their primary biomolecules [56]. In addition, circRNA and lncRNA were found in the exosomes [57].

2.2.2.2 Biological composition of plant-derived exosomes

Each plant species has a different influence on the exosome makeup. As a result, bioactivity and functions like absorption, targeting, and altering gene expression are species-specific. [15, 17].

a) Proteins

The protein content in plant-derived exosomes is relatively low [22]. The proteomic analyses of ginger exosomes have established chloride channel proteins and aquaporins [58]. Proteomic studies on *N. tabacum* revealed that the 40 S ribosomal proteins S6, S4, and an 85 KDa hydrolase ß-xylosidase/alpha -L-arabinofuranosidase 2 related protein were exosome-specific proteins [59]. A lack of comparative databases, contamination by numerous enzyme proteins as RuBisco, and the fact that abiotic and biotic stresses significantly affect the proteome content of plant exosomes, are all factors that limit proteomic studies [22, 59].

b) Secondary metabolites

Curcuminoids and chlorophylls, which are lipophilic compounds, were found to make up the majority of the exosomes from a number of plants [22, 39]. Studies on plant exosome secondary metabolites can often be influenced by the acidic/basic conditions of the extraction buffering system [59]. The anti-microbial activity and rise in its synthesis during infections are explained by the existence of secondary metabolites in the exosomes [22, 39].

c) Lipids

Predominantly, plant-derived exosomes contain phospholipids [22]. Numerous phospholipids were identified by HPTLC studies, and they are essential for the stability of nanovesicles and for cell-specific targeting [39]. Exosomes' lipid components work as signals for specific gut bacteria to selectively absorb them [60]. Phosphatidic acid lipids regulate the *Lactobacillaceae* bacteria's ability to take these particles up and sustain their duration and accumulation in the gut [39].

d) miRNAs

By precisely attaching to and cleaving the target mRNAs or by preventing translation, microRNAs can control gene expression [61]. The cross-kingdom function of plant exosomes

are carried out by these short non-coding RNAs, which are an essential component of the exosomal active components in plants [61, 62]. Exosome delivery facilitates the uptake of miRNAs from dietary sources. Research indicates that dietary miRNAs can enter the body and regulate human genes [63]. A study to determine the miRNAs linked to edible plant-derived exosomes found 418 miRNAs, with each species' miRNAs ranging from 32 to 127 [61]. These miRNAs' functional assays and target prediction revealed genes from cancer-related pathways and the inflammatory response [61]. In a recent study on exosome associated miRNAs in ginger, it was discovered that there are 27 miRNAs that are substantially expressed and target several cytokine genes involved with inflammation [22, 64]. Additionally, a number of studies suggest that exosomes target the consuming organism's digestive system and have the ability to regulate physiological processes via miRNAs [64].

The table below **(Table 2.1)** highlights the differences between the biological composition of plant-derived and mammalian-derived exosomes.

Table 2.1: *The biological composition differences between the plant-derived and mammalian-derived exosomes.*

Parameters	**Plant-derived exosomes**	**Mammalian-derived exosomes**	**References**
Size (nm)	30-400	30-150	[65, 66]
Zeta Potential (mv)	-[1]70 to neutral	-34.3 to −6.3	[67]
Proteins	More membrane transporters, such as chloride channels and aquaporins, than cytosolic proteins, such as proteolysis enzymes and actin.	Contains molecules from the major histocompatibility complex, fusion proteins, cytoskeletal proteins, membrane transporters, chaperones, proteins involved in multivesicular body production, and the tetraspanin protein family.	[58, 68]
Lipids	Consists of digalactosyl monoacylglycerol,	Contains cholesterol, sphingomyelin,	[67, 58, 69]

[1] – indicates a negative value, i.e. a value less than zero.

	digalactosyl diacylglycerol, phosphatidic acid, phosphatidylethanolamine, phosphatidylcholine, and monogalactosyl diacylglycerol.	glycosphingolipids, and phosphatidylserine.	
Nucleic Acids	Contains non-coding RNA, mRNA, miRNA and Deoxyribonucleic acid (DNA).	Contains DNA, mRNA, miRNA, mitochondrial DNA, and lncRNA	[60, 70]
Means of internalization	Occurs via phagocytosis, macropinocytosis, as well as endocytosis mediated by clathrin.	Occurs via endocytosis and phagocytosis.	[71]
Common route of administration	Oral.	Intravenous.	[58, 65]
Safety	The safety of non-gastrointestinal administration still needs to be investigated further.	Established to be relatively safe.	[58, 65]

2.2.3 Therapeutic applications

2.2.3.1 Medicinal and clinical properties of mammalian exosomes

Over the last decade, the clinical use of exosomes as diagnostic biomarkers and drug delivery vehicles have emerged as a significant research area, sparking an increase in clinical trials, funding, and academic research [18, 31].

The following are some of the main exosome properties relevant to therapeutic and diagnostic uses: Exosomes are nano-sized, released, and taken up by almost all cells in the body. They are also essential for cellular communication and regulation, have cellular markers that can target particular cell types, are non-immunogenic, more stable, and easier to handle, and carry proteins, RNAs, DNAs, and lipids that can be endocytosed by nearby or distant cells and modulate the recipient cells [17] [72].

a) Biomarkers

Studies have shown that cells that are going through an inflammatory phase emit more exosomes [73]. The current trends on exosome studies are focused on liquid biopsies [72].

Liquid biopsies are more accessible, less invasive, provide a more general representation of the tumour, and allow for serial monitoring [72]. Liquid biopsies make it simple to obtain miRNAs in biological fluids. As non-invasive diagnostic biomarkers, a number of exosomal miRNAs are being developed, such as miRNA-21 for oesophageal squamous cell cancer [18]. Exosomes discharged in pathological situations contain constitutive elements, such as transmembrane proteins or nucleic acids. These may serve as biomarkers for clinical diagnosis, staging the severity of a disease, or evaluating a therapy response [18].

Tetraspanins, which are frequently present at the exosomal membranes, include CD9, CD63, CD81, and CD151. These can be utilised as screening techniques [18]. Exosome-associated tau phosphorylated at Thr-181 and exosome-aggregation-prone protein amyloid peptide are recognized diagnostic indicators for Alzheimer's disease in neurodegeneration [74]. For early-stage Parkinson's disease, autolysosomal proteins such cathepsin D, LAMP, or synuclein have been considered as diagnostic indicators [75].

Salivary exosomes have been shown by researchers Boyzk *et al*. (2023) to be a prospective biomarker for the early detection of oral squamous cell cancer [76]. According to the study, the protein panel PSB7, AMER3, and LOXL2 successfully distinguished between cancer patients and healthy individuals with an area under the curve (AUC) of 0.96, 100% sensitivity, and 75% specificity [76]. In a study conducted by Welker *et al*. (2012), it was discovered that CD81 is elevated in the exosomal serum fraction in people with chronic hepatitis C and is connected to inflammation and the severity of fibrosis [77]. CD81, a tetraspanin, is a key HCV hepatocyte cell entrance receptor. Following differential centrifugation, the amount of soluble CD81 was semi-quantitatively measured using immunoblotting and densitometry [77].

b) Drug delivery

Drugs such as Adriamycin® can be administered directly to tumour cells by utilizing the target-specific characteristic of exosomes, which lowers their toxicity to healthy cells [78]. Studies have demonstrated that the exosomes produced from mesenchymal stem cells and several other cells in cell therapy are responsible for the cellular immunity, angiogenesis, and regenerative effects. This opens the door to ‘cell-free cell therapy’ [78].

Exosomes further have the potential to be utilised as vaccine carriers [78]. The potential of using exosomes to deliver vaccines has been explored and they have been tested by delivering hepatitis C-associated antigens to mice primed with serotype 5 recombinant adenovirus vectors (rAd5) [79]. This also included the use of extracellular vesicles that came from diseased cells to treat infectious disorders. Dendritic cell exosomes, primed with *Toxoplasma gondii* antigens, were efficacious in mediating a repressive immunological response [79]. The SARS-CoV-2 coronavirus was then evaluated using this strategy *In vitro*.

The viral spike S protein was loaded in the exosomes [80]. With regards to COVID-19, numerous exosome-based investigations and clinical trials have been initiated. Trial NCT04276987, sponsored by Ruijin Hospital in Shanghai and Jiao Tong University School of Medicine, is an example of such [18, 81]. For these ongoing trials, there are no efficacy or safety outcomes available yet [18].

Exosomes have been recommended as targeted delivery systems for the treatment of ischemic strokes in a study conducted by Tian *et al*. (2018) [82]. The mesenchymal stromal cell-derived exosomal surface was coupled with the c(RGDyK) peptide. When administered intravenously, curcumin-loaded c(RGDyK)-coupled exosomes in the temporary middle cerebral artery occlusion mouse model, specifically targeted the lesion region of the ischemic brain [82]. The results showed that the inflammatory response and cellular death were significantly reduced in the lesioned area [82]. Short interfering (si)RNA can be delivered to the brain of mice to treat Alzheimer's disease using exosome-endogenous nano-vesicles that transport RNAs and proteins. This was shown in a study by Alvarez-Erviti *et al*. (2011) [83]. Exosomes were loaded with siRNA and administered intravenously to neurons, microglia, and oligodendrocytes in the brain, knocking down a specific gene. Strong knockdown of BACE1 by 60% mRNA and 62% protein, a target for treating Alzheimer's disease, confirmed the therapeutic potential of exosome-mediated siRNA delivery [83].

c) Reproduction

Steroid synthesis, oocyte maturation and granulosa cell proliferation can be regulated by exosomes in the follicular fluid that transport proteins, miRNAs, and mRNA [23]. By using the PI3K/mTOR signalling pathway, Yang *et al*. (2020) demonstrated that miR-21-5p or miR-146a-5p exosomes from human umbilical cord mesenchymal stem cells improved mice oocyte quantity and quality, accelerated oocyte development, and increased oocyte production [84].

Exosomes are known to regulate implantation, embryo development and follicle development and are secreted by the endometrium, oviduct epithelium, uterus, and preimplantation embryos [49]. Exosomes are released by the endometrium into the fluid of the uterine cavity to control the embryo attachment process. During embryo implantation, the embryo itself can produce exosomes. Exosomes released by the embryo change the endometrium's shape and gene expression profile, controlling the invasion and positioning of the embryo for implantation [23]. Exosomal proteins and miRNAs in the placenta are also known to control trophoblast invasion and the inflammatory response. Exosomes from the uterine and embryonic mucosa have the functional ability to mediate immunological tolerance, enabling embryo implantation and subsequent pregnancy in the mother. Recent research has revealed that the maternal immune system is downregulated by EV-derived miR-98 and bta-miR-26b, which aids in the

conceptus' implantation [23]. Additionally, exosomes from testicular cells control healthy steroidogenesis and spermatogenesis in the testis and may be employed as a diagnostic indicator for male infertility [23].

The maturation of sperm has been linked to seminal plasma exosomes [49]. Exosomes produced from seminal plasma inhibit HIV-1 infection as well. This can be attributed to the prevention of HIV-1 early protein transcriptional activator recruitment and subsequent transcription [49]. Male seminal plasma exosomes included 1,474 proteins, according to Yang *et al*. (2017). These proteins were found to play a major role in protein metabolism, energy pathways, metabolism, as well as cell growth and maintenance [85].

By delivering exosomal miRNA from the placenta's specialized trophoblast cells to nonplacental cells, exosomes can help prevent infections of the placenta caused by viruses like poliovirus, human cytomegalovirus, and herpes simplex virus 1 [49]. Exosomes that are specific to a particular gestational stage develop during pregnancy and are functionally connected to labour and delivery [49].

Exosomes in breast milk support postnatal growth and health. The exosomes increase the *ex vivo* production of peripheral blood-derived T-regulatory cells and include miRNAs with immune-related activities [49]. This may aid to control immunological tolerance [49].

d) Therapeutic agents

The primary sources of therapeutic mammalian exosomes, whether natural or manufactured, include dendritic cells, autologous tumour cells and mesenchymal stem cells.

(i) Cancer therapy

Patients participating in the oncogene inhibition trial are injected with exosomes loaded with siRNA specific to the KrasG12D oncogene. Its expression is decreased in pancreatic tumours as a result [18]. The advantage of using a patient's own tumour cells as manufacturing cell lines, is that it prevents neutralisation via innate immunity.

Research by Du *et al*. (2014) has demonstrated that human Wharton's jelly-derived mesenchymal stem cell exosomes promote the proliferation and invasion of human renal carcinoma cells [86].

According to Hong *et al*. (2018), bone marrow mesenchymal stem cell exosomes can carry siRNA-GRP78 to cancer cells of the liver and reduce the GRP78 expression, making the cancer cells more susceptible to Sorafenib® [87].

(ii) Anti-inflammation/immunomodulation therapies

Studies have demonstrated that exosomes can be employed for neurological therapies as therapeutic agents that specifically target the brain [18]. Exosomes with an engineered c(RGDyK) conjugation were able to target an ischemic brain injury. Resolvin D2 loaded neutrophil membrane-derived exosomes were proposed by Dong *et al*. (2019) to be carried to the brain, specifically to a stroke lesion, where they would operate as an anti-inflammatory drug [88].

Umbilical cord blood mesenchymal stem cell exosomes were used by Nassar *et al*. (2016) to study their impact on β-cell mass type 1 diabetes mellitus [89]. By boosting the amount of certain regulatory T cells lymphocytes, the stem cells have successfully modulated the autoimmune response against β-cells, re-establishing the Th1/Th2 immunological balance [89]. No results from the phase 1 trial have been disclosed yet.

Patients suffering from graft versus host disease were administered with mesenchymal stem cell-derived exosomes, as shown by Kordelas and co-workers (2014). From their findings, it was demonstrated that the pro-inflammatory cytokine response was decreased [90].

2.2.3.2 Therapeutic potential of plant-derived exosomes

Numerous I*n vitro* and *In vivo* investigations have documented the ability of plant-derived exosomes to treat a variety of biological conditions [22]. **Figure 2.2** illustrates the various therapeutic benefits of plant-derived exosomes.

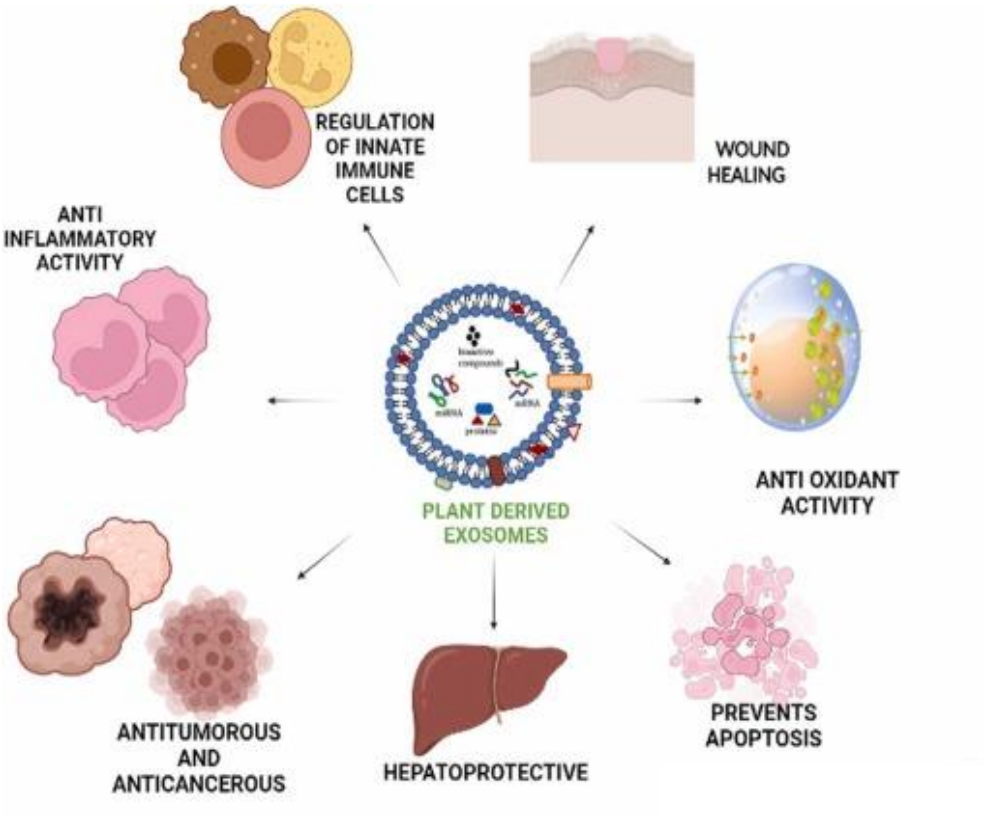


Figure 2.2: *Different therapeutic advantages of plant-derived exosomes* [22]. *As illustrated, plant-derived exosomes can be utilised for their anti-oxidant, anti-tumourous, anti-cancerous and anti-inflammatory properties. Further, plant-derived exosomes are also known for their wound healing and hepatoprotective abilities. They are also seen to play a role in the regulation of innate immune cells and in apoptosis prevention.*

The table below summarises the reported therapeutic activities of plant-derived exosomes from different dietary sources.

***Table 2.2**: Therapeutic activity/potential of plant-derived exosomes from different dietary sources.*

Source	Therapeutic activity/potential	References
Apples, grapes, cabbage, ginger, broccoli, garlic, grapefruit, *Lobostemon fruticosus*	Anti-inflammatory	[91, 67, 92, 93, 94, 95, 96, 58, 97, 98]
Strawberry, grapefruit, ginger, blueberry, bitter melon, carrot, aloe vera, c*itrus limon*	Antioxidant	[99, 96, 39, 100, 101, 36, 38]
Grapefruit, aloe vera, wheat	Wound healing	[102, 103, 38]
Grapefruit, lemon, tea flowers, garlic	Anti-cancer	[96, 104, 105, 106]
Ginger	Hepatoprotective	[39]
Blueberry, bitter melon, ginseng*, grapefruit, Citrus limon, ginger*	Anti-tumour	[36, 39, 107, 100, 101, 35]
Garlic	Anti-obesity	[108]
Lemon, ginger, coconut water	Anti-microbial	[104, 39]
Wheat grass juice, aloe vera, c*itrus limon*	Restorative	[36, 38, 102]

(i) Antioxidant properties

Antioxidant compounds, found in abundance in plants, are frequently used for the creation of anti-aging formulas and for food preservation.

Cardiomyoblast and neuroblastoma cell lines were used to investigate the antioxidant capacity of Carex™, a nanovesicle made from carrots [109]. In both SH-SY5Y neuroblastoma cells and H9C2 cardiomyoblasts, Carex™ showed a low level of cytotoxicity. Additionally, Carex™ greatly reduced the production of reactive oxygen species (ROS) and apoptosis *In vitro* in models of Parkinson's disease and myocardial infarction. In both models, Carex™ prevented a decrease in the expression of antioxidant molecules, Nrf-2, HO-1, and NQO-1 [109].

Zhao *et al*. (2022) discovered that high-fat diet fed C57BL/6 mice and rotenone-induced HepG2 cells could both benefit from the antioxidant properties of blueberry-derived exosomes-like nanoparticles [100]. Exosome treatment of HepG2 cells resulted in a decrease in ROS, an increase in mitochondrial membrane potential, and a decrease in apoptosis [100]. The exosomes reduced liver dysfunction in the mice that were subjected to a high-fat diet. A drop in aspartate transferase and alanine transaminase levels was also seen [100]. By inhibiting the enzyme fatty acid synthase and acetyl-coA carboxylase, the blueberry exosomes decrease lipid droplet production [100].

Citrus limon exosomes have demonstrated antioxidant properties that can be attributed to the presence of ascorbic acid as one of its constituents. Human mesenchymal stromal cells easily take up the exosomes, without any harmful effects. These cells exhibit much better survival when exposed to Hydrogen Peroxide (H_2O_2) in a dose-dependent manner. Additionally, these cells produced far less ROS [36]. According to a study done by Baldini *et al*. (2018), exosome-like nanovesicles from Citrus limon juice contain measurable levels of citrate and ascorbic acid. *In vitro*, these exosomes were taken up by human mesenchymal stromal cells and exhibited a strong antioxidant impact [36]. As a well-known cofactor for numerous significant enzymatic activities and a well-known ROS scavenger, ascorbic acid guards against apoptosis caused by oxidative damage [36]. Exosomes from berries and fruits heavily concentrated with ascorbic acid can endure the tough gastric environment and have improved intestinal absorption. This ensures that a significant concentration of ascorbic acid is present and is successfully delivered to the target cells. For instance, 50 g/mL citrus limon exosomes contains approximately 6.47 μM of ascorbic acid. This is equivalent to a concentration that is around 40 times lower than what is required to experimentally prevent cells from dying from H_2O_2 -induced oxidative stress [36].

(ii) Anti-tumorigenic property

Numerous studies have observed the anti-neoplastic properties of exosomes, and these findings may be utilised to develop safer, natural cancer therapy regimens [14, 22]. Most traditional cancer therapies also have a similar strength of impact on normal and healthy cells. This leads to the many adverse side effects experienced [22]. Nanoparticles sourced from plant sources do not cause adverse effects as plant-derived exosomes are selective towards and target malignant tumour cells [14, 22].

Numerous studies have highlighted the potential and benefits of using *Cannabis sativa* extracts and its phytocannabinoids in the treatment of cancer [110]. Tajik *et al*. (2022) isolated two chemotypes of cannabis and they were divided according to their cannabidiol (CBD) content. EVs were categorised as high-CBD (H.C-EVs) or low-CBD (L.C-EVs) EVs.

Cytotoxicity studies revealed that Huh-7 and HepG2 hepatocellular cancer cell lines were significantly less viable when exposed to H.C-EVs in a dose- and time-dependent manner [110]. The results demonstrated that the H.C-EVs substantially triggered cell death by the activation of the mitochondrial-dependent apoptosis signalling pathways in both HCC cell lines, stopping the G0/G1 phase of the cell cycle [110].

In a study by Quesenberry *et al*. (2023), exosomes from grapes, grapefruit, ginger, and carrots have been shown to decrease the growth of the chronic myeloid leukaemia xenograft model *in vivo* [17]. The apoptotic mechanism of TNF-related apoptosis-inducing ligand, by which these nanoparticles exhibit their anticancer effect, was demonstrated [17].

Exosomes from the tea plant *Camellia sinensis,* demonstrated potent cytotoxicity in breast cancer cells [106]. They induced oxidative stress in cancer cells, which resulted in mitochondrial damage and cell-cycle arrest. Breast cancer cells were given anti-migration, anti-proliferation and anti-invasion actions I*n vitro* as a result of ROS amplification [106]. To evaluate the *in vivo* effect, xenograft mouse models were used. It was found that either oral or intravenous dosing increased the survival rate [106]. With oral administration, the exosomes via the intestinal epithelial cells, were absorbed into the blood [106]. By altering their proliferation, apoptosis, and motility, they aggregated in the breast cancer cells and stopped metastasis to the lungs. Additionally, the exosomes' capacity to control the quantity and variety of microbiota resulted in some advantageous outcomes from oral delivery [106].

On tumour cells, exosomes from the plants *Dendropanax morbifera* and *Pinus densiflora* had cytotoxic effects [111]. Phagocytosis and caveolae-mediated endocytosis, which is prevalent in tumour lines, are used to take up these exosomes. Therefore, compared to normal cells, they accumulate more strongly in tumour cells. The exosomes' ability to suppress growth is what is responsible for their therapeutic effectiveness [111].
When co-administered, *Dendropanax morbifera* and *Pinus densiflora* exosomes significantly increased the suppression of cancer cells [111]. They are regarded as a promising treatment regimen in treating skin and breast cancers with minor adverse effects [111]. Kim *et al*. (2020) further explored the anti-cancer properties of *Dendropanax morbifera* exosomes using a 3D microfluidic model simulating the tumours microenvironment [112]. The fibroblasts associated with cancer were suppressed by the exosomes in a concentration-dependent manner. ECM-related proteins such as collagen, and a few growth factors were affected by the exosome treatment in terms of expression levels [112].

In some tumour cell lines, grapefruit exosomes have demonstrated a 40% reduction in viability [96]. In a study by Stanly *et al*. (2020), melanoma cell lines were used showed that the therapeutic potential of grapefruit is attributed to the cell cycle being arrested at the G2/M

check point, which results in a decrease of cycB1 and cycB2 cyclins and an increase of the cell cycle inhibitor P21 [96]. CycB2 is downregulated to reduce invasiveness and stop metastases. Additionally, the grapefruit exosomes reduce the ability of cancer cells to proliferate by inhibiting the ERK and AKT signalling pathways and demonstrating pro-apoptotic activity by activating PARP-1 [96]. The grapefruit-derived exosomes' metabolome profile showed that they are particularly rich in alpha-hydroxy acids, leucine/isoleucine, myo-inositol, and the anti-proliferative substance doconexent, demonstrating its anticancer activities [96].

Exosomes from citrus limon significantly slow down, by 50%, lung cancer growth and chronic myeloid leukaemia cell lines [22]. Additionally, exosome therapy upregulates pro-apoptotic genes such as Bax and Bad, while downregulates anti-apoptotic genes such as Bclx-1 and Survivin. TNF-related apoptosis inducing ligand (TRAIL) is a naturally occurring mechanism for immune defence against viruses and cancer cell immunosurveillance [113]. Within 48 hours of treating the cell lines, the mRNA levels of TRAIL and its receptor Dr5 were both increased. Exosomes inhibited the release of cytokines that promoted angiogenesis and activated TRAIL-mediated apoptosis in xenograft tumour models, resulting in a decrease in tumour growth *in vivo* [113].

Orally administered ginger exosomes significantly reduced tumour sizes in chemically generated colorectal cancer mice by inhibiting apoptosis, IEC proliferation and pro-inflammatory cytokine production [58].

Zeng *et al*. (2021) suggested using an Aloe vera-derived nanocarrier for cancer treatment [114]. The isolated nanovesicles showed strong structural and storage stability, as well as antioxidant and anti-detergent capabilities. Without exerting toxicity I*n vitro* or I*n vivo*, they may be readily absorbed by melanoma cells [114]. The nanovesicles were loaded with indocyanine green, which demonstrated excellent stability with a retention rate of more than 90% after 30 days of storage. Nanovesicles were still capable of harming and suppressing the growth of melanoma cells after 30 days [114].

(iii) Restorative property

Exosomes from citrus limon improve type I collagen synthesis [36]. Citrate, a micronutrient found in citrus juice, plays a crucial role in bone homeostasis regulation and in the mediation of the bone matrix collagen structure. Citrate and a mixture of other bioactive chemicals found in exosomes are thought to be responsible for this action [36].

Exosomes from Aloe vera regulate Nrf2 to activate the antioxidant defence and prevent oxidative damage in a dose-dependent way [38]. According to an I*n vitro* scratch wound study,

exosomes encourage keratinocyte and fibroblast migration to the wound site, which aids in wound healing [38].

On epithelial (HaCaT), endothelial (HUVEC) and dermal fibroblast (HDF) cells, it has been demonstrated that exosomes derived from wheat grass juice have strong proliferative and migratory effects [102]. Using I*n vitro* methods, Sahin *et al*. (2019) demonstrated these effects on the HaCaT, HUVEC and HDF cells [102].

(iv) Anti-inflammatory property

According to several studies, plant-derived nanoparticles can reduce the signs and symptoms of inflammation [14, 22, 33].

According to a study by You *et al*. (2021), consumption of cabbage and red cabbage-derived exosome-like nanovesicles reduced the expression of COX-2, IL-6 and IL-1b in macrophages that had been exposed to lipopolysaccharide, demonstrating a definite anti-inflammatory impact [92]. Likewise, Raimondo *et al*. (2022) isolated exosomes from citrus limon juice and identified the limonoid, lipid and flavonoid contents of these exosomes. The results showed that in LPS-stimulated murine macrophages, these EVs inhibited NF-κB nuclear translocation and phosphorylation. Further, the gene and protein expression of pro-inflammatory cytokines including IL-6, IL1-β and TNF-α were suppressed [115]. In addition, *ex vivo* studies using human primary T cells demonstrated that lemon-derived exosomes can reduce pro-inflammatory cytokines and enhance anti-inflammatory chemicals [115].

Exosomes from onions were extracted by Kang and colleagues (2022), who then looked at how well they reduced inflammation in RAW 264.7 macrophages [116]. In RAW 264.7 cells, these EVs demonstrated a dose-dependent inhibition of pro-inflammatory cytokine production and expression, including cyclooxygenase 2, inducible nitric oxide synthase, interleukin 6 and interleukin 1β [116]. Additionally, the phosphorylation of NF-κB, a transcription factor that controls the production of other pro-inflammatory proteins, is altered by onion EVs [116].

Yang *et al*. (2021) showed that EVs made from bitter melon have inherent anti-inflammatory properties [117]. The EVs caused apoptosis and an inhibition of the S phase cell cycle. ROS generation and JUN protein overexpression were required for the induction of apoptosis. Furthermore, NLRP3 expression was noticeably downregulated [117].

Ginger exosomes were administered orally to colitis mice models, and the results show that neither local nor systemic side effects occurred after their efficient absorption by macrophages and epithelial cells [58]. Studies conducted *In vitro* and *in vivo* by Zhang *et al*. (2016) revealed considerable mucosal wound healing owing to the exosome nanoparticles. By decreasing pro-inflammatory cytokines like TNF-α, IL-6, and IL-1β and increasing anti-inflammatory cytokines

like IL-10 and IL-22, they further improved the survival and proliferation of intestinal epithelial cells [58]. Ginger, which contains gingerol, has anti-inflammatory and anti-infective properties [32]. Strong anti-inflammatory effects in carrot juice help to alleviate the signs and symptoms of arthritis and rheumatism [32]. By stimulating stem cell proliferation, grape exosomes encourage the self-renewal of intestinal epithelium [118]. Exosomes extracted from *Citrus sinensis* fruit juice increased permeability-related genes while downregulating many inflammatory genes. By modifying the tight junction OCLN protein's distribution, they reduce inflammation and enhance the integrity of the intestinal mucosa [119]. *Citrus sinensis* EVs were confirmed by Bruno *et al*. (2021) to be stable in the gastrointestinal environment and were readily absorbed by intestinal cells without exerting any adverse effects [119]. Additionally, it was shown that *Citrus sinensis* EVs can modify the gene expression of a number of inflammation-related genes, helping to reduce inflammatory stimuli and re-establish a functional barrier [119]. In response to TNF-α, exosomes from blueberries alter the expression of approximately 29 genes implicated in inflammatory pathways in human endothelial cell lines [100]. Blueberry exosomes' miRNA content revealed potential mRNA targets associated with the inflammatory response, oxidative stress and cytokine release. This demonstrates that bioactive components with potent anti-inflammatory effects are therapeutically delivered by blueberry exosomes [100]. Through the activation of adenosine monophosphate activated protein kinase (AMPK), it was discovered in a study by Deng *et al*. (2017) that exosomes derived from broccoli lower inflammation by increasing dendritic cell tolerance [97]. The activation of the AhR signalling pathway is promoted by exosomes from the edible bark of mulberries [120]. It has been demonstrated that apple exosome preparations have anti-inflammatory properties, which are mostly due to M2 macrophages' production of the miRNA146 [120].

(v) Anti-microbial property

Porphyromonas gingivalis specifically absorbs exosomes derived from ginger, lowering its virulence [108]. According to research published by Sundaram *et al*. (2020), *Porphyromonas gingivalis* selectively absorbs ginger exosome nanoparticles in a phosphatidic acid-dependent way through interactions with hemin-binding protein 35 on the surface of the organism [108]. Exosomal lipids, anti-microbial peptides, and miRNAs target several *Porphyromonas gingivalis* virulence factors to decrease the pathogenicity of the organism [108].

According to a study by Yu *et al*. (2019), coconut water exosomes can penetrate *Lactobacillus plantarum* WCFS1 and *Escherichia coli* K-12 MG1655 cells after co-incubation for one hour [121]. The miRNAs found in the exosomes of coconut water, like miR-395, may have potential targets in the bacteria [121]. Exosomes have the ability to change the expression of bacterial

genes because of the cross-kingdom transfer of miRNAs. This suggests that exosome ingestion may affect how bacteria survive and invade [121].

By fostering the development of mucus and proteins related to the intestinal barrier, *lactobacilli* defend the intestinal barrier from infections and prevent intestinal damage [122]. Additionally, they release antimicrobial compounds like SCFAs, bacteriocins, and hydrogen peroxide. These prevent pathogen growth and eradicate it [122]. *Lactobacillaceae* preferentially absorb ginger-derived exosomes to aid in their development [60]. Due to the altered microbiome and their miRNAs targeting LGG genes, these exosomes indirectly improve the function of the intestinal barrier and prevent mouse colitis [60]. The *Runococcaceae* family particularly absorbs exosomes from grapefruit. This suggests that dietary-derived grapefruit exosomes have a distinct composition and that the gut microbiota changes dramatically depending on the individual diet [60].

Lemon-derived exosomes boost the survival of *Streptococcus thermophilus ST-21* and *Lactobacillus rhamnosus GG* by improving their bile resistance in the gut [123]. These exosomes have the capacity to improve probiotic survival and change the composition of intestinal metabolites, which can prevent the *Clostridioides difficile*-induced colitis [123]. Exosomes modify the intestinal milieu to prevent the growth of *Clostridiodes difficile*, although not being directly bactericidal [123].

(vi) Plant-derived exosomes as a drug delivery system

Adverse effects are frequently caused by prolonged usage of conventional medication therapies [30]. Plant-derived exosomes have demonstrated their innate capacity to transport chemical compounds [17]. They have excellent biochemical characteristics [30], including a good biocompatibility [124], low toxicity and immunogenicity [125], target-specific ability [125], the lengthening of the drug's cycle and its duration of action [124], ability to produce in high numbers and to penetrate the blood-brain barrier without passing through the placenta [24]. Furthermore, exosomes can shield their contents from external agents due to their lipid, plasma membrane structure [17]. The capacity to modify genes for therapeutic purposes, transmit hydrophobic medicines, and evade immunological responses make plant-derived exosomes excellent candidates for drug delivery vehicles. Studies have revealed that plant-derived exosomes can successfully carry a variety of chemicals and nucleic acid drugs, thereby decreasing inflammation or suppressing gene expression [126]. Using a biomimetic nanocomposite, Mao *et al*. (2021) administered anti-TNF-α antibodies orally to colitis mice to demonstrate the excellent drug loading capacity of ginger-derived exosomes. Compared to intravenous treatment, a high effectiveness and a decrease in severe systemic adverse effects were observed [127]. Similar to this, Zhang and colleagues (2016) developed a nanovector

from lipids obtained from ginger that acted as a platform for the delivery of doxorubicin in colon cancer treatment [128]. Using the ginger, they created nanoparticles. Thereafter, the reassembled lipids from the ginger resulted in ginger-derived nanovectors. Results revealed that nanovectors were efficiently taken up by colon cancer cells [128].

Plant-derived exosome delivery has advantages over conventional approaches, including increased drug absorption rates, site-specific administration, excellent biocompatibility, and non-toxic adverse effects [30]. Exosomes are able to meet the strict standards of clinical application owing to the fact that they are effective drug nanocarriers and have distinctive morphology and compositional traits. They do not cross the placental barrier when given intravenously to pregnant mice, demonstrating their potential as an effective medication delivery method [17, 129].

2.2.4 Stability of exosomes

Exosomes have the potential to improve the bioavailability of bioactive chemicals and have been shown to increase the stability of their own content [130]. Research conducted by Mandal (2019) shows that exosomes are resistant to both digestive enzymes and those present in other body fluids [130]. As a result, preservation against degradation is made sure until they reach their destination [130]. Exosomes, as opposed to ectosomes and apoptic bodies, have a liquid-ordered phase membrane and are less sensitive to detergent lysis, according to Osteikoetxea *et al.* (2015) [131]. According to a study by Mandal (2017), exosomes made from EL-4 cells contain curcumin that is approximately four times more stable and more water soluble than free curcumin [132].

2.2.4.1 Stability of mammalian exosomes

When newly isolated, exosomes are at their optimal state. However, at -80°C, exosomes can be stored for up to one year without coagulating [130]. It should be highlighted that exosomes' structural integrity and shelf life are impacted by repeated freeze-thaw cycles [130]. Exosome-containing biological fluids can be kept at 4°C in a glass bottle for up to five days. Additionally, stability at 20°C for up to 3 months has been recorded [130]. Despite being stable at low temperatures, pressure, multiple freeze-thaw cycles, the type of solvent used, and the length of storage could have an impact on the structural and physicochemical characteristics of mammalian-derived exosomes [18, 72].

2.2.4.2 Stability of plant-derived exosomes

Different biological conditions result in plant-derived exosomes behaving differently [130]. Exosome size and surface charge variations are influenced by the environment and plant source. Exosome characteristics, for instance, might be pH-dependent. When compared to exosomes suspended in water, grape-derived exosomes shrank in size in the stomach and intestine [130]. However, in settings mimicking those found in the stomach and intestine, ginger-derived exosomes grew in size [130].

As an alternate drug delivery system, the stability of the carrier is essential since it can impact drug absorption and distribution *in vivo* [114]. Utilizing the antioxidant and anti-detergent properties of aloe, Zeng *et al*. (2021) examined the stability of the aloe-derived nanovesicles [114]. The antioxidant potential of aloe-derived nanovesicles was evaluated using the oxygen radical absorbance capacity assay. At a concentration of 1×10^8 particles/mL, the results demonstrated an approximate 100% recovery. The non-ionic surfactant Triton X-100 (TX-100) was utilised as a membrane breaker to examine the anti-detergent capability. The unimodal size distribution of aloe-derived nanovesicles was maintained at a concentration of 0.1% TX-100 [114]. The apex of the size distribution of the aloe-derived nanovesicles decreased with increasing TX-100 concentration, although it remained constant at 2% TX-100 concentration. The various phospholipids in aloe-derived nanovesicles, according to the researchers, allowed for a more structured phospholipid arrangement and hindered the insertion of TX-100 molecules into lipid bilayers. This allowed for structural stability, whilst limiting the formation of the vesicles-detergent mixed micelles and alleviated dissolution [114].

2.3 Lobostemon fruticosus

Commonly referred to as the ‘agtdaegeneesbos’, *Lobostemon fruticosus* has been traditionally used as a medicinal plant in South Africa [94]. This directly translates in Afrikaans to ‘eight-day healing bush’ - claiming that the plant simply can heal a condition in a mere eight days [93]. Other commonly used vernacular names of this plant include the pyjama bush, Douwurmbos (dew worm bush), Luibos (lazy bush) and Geneesbos (healing bush) [93, 94, 95, 133]. The leaves and stems are used to treat a variety of conditions, including wounds, skin diseases and gynaecological disorders [12, 134, 7, 8]. *Lobostemon fruticosus* belongs to the Boraginaceae family [95]. Plants belonging to this family are well known for containing pyrrolizidine alkaloids [135, 136], terpenoids [137], glycosides [137] and flavonoids [137], which exhibit diverse range of bioactivities [37]. In their analysis of the phytochemical components of the leaves and twigs of *Lobostemon fruticosus*, Bedane *et al*. discovered a total of 13 compounds [138]. Rutin, *p*-hydroxybenzoic acid, lycopsamine-N-oxide, rosmarinic acid, caffeic acid, loliolide, syringaresinol, 3-indolcarbaldehyde, pinoresinol, rabdosiin, keampferol-3-*O*-rutinoside globoidnan B and globoidnan A were among the substances found.

The levels of the cytokines IL-1, IL-2, IL-6, GM-CSF and TNF- in the supernatant medium of the human peripheral blood mononuclear cells activated by lipopolysaccharide were used to measure the anti-inflammatory activity. Rabdosiin, rosmarinic acid and syringaresinol significantly decreased the secretion of IL-1β, IL-2 and IL-6. TNF-alpha production was significantly decreased by all 13 compounds [138]. Additionally, according to traditional healers, the plant has anti-HIV properties [94]. The pharmacological research has revealed its anti-bacterial [139], anti-viral [139], anti-proliferative [140] and cytotoxic activities [141], making it an ideal candidate for therapeutic use.

Given all the therapeutic benefits of this plant, the aim of this research project was to investigate the wound healing potential of exosomes derived from *Lobostemon fruticosus*.

2.4 Conclusion

Drug development using nanotechnology has gained tremendous popularity over the last decade and is now successful in several therapeutic areas such as cancer therapy agents for chemotherapy, disease biomarkers as well as drug and vaccine carriers. Mammalian- and plant-derived exosomes have demonstrated to be viable candidates to be utilised in nano-based medicines. Over the last decade, the relevance and importance of exosomes therapeutically and clinically have been established. As a result, research on exosomes is undergoing a period of rapid growth. The growth has been associated with exosomes' abilities to act as drug carriers, disease biomarkers, therapeutic agents – to name but a few. Despite recent developments, there remains several issues of exosome utilisation that warrant further research and clarification. A thorough understanding of their biology is fundamental in utilising exosomes clinically. Additionally, a number of techniques are being developed to maximize the therapeutic effectiveness of exosomes. The regulatory environment is changing to support safe and effective clinical trials investigating the clinical use if exosomes. To overcome the restrictions of manufacturing and characterization procedures, better scaling up solutions are being developed. Hence, whilst all the efforts are still at the early stages, the exosome field is rapidly advancing towards successfully approved commercial use. Due to their unique physiological, chemical and biological properties, exosomes offer a great deal of potential for therapeutic applications in disease therapy as well as in the creation of nanocarrier drug delivery systems that can deliver a range of dosages. If diseases are to be treated more efficiently and successfully, advancements in the use of cutting-edge, innovative drug development approaches are needed. For millennia, people have been using plants and natural products for their anti-microbial, antioxidant, and anti-inflammatory properties. It is for this reason that plant-derived exosome-based therapies have the potential to be a novel method in the treatment of malignancies, inflammation, immune-related disorders and a

variety of other medical conditions. More therapeutic benefits have been established, with less adverse effects. Hence, the move away from conventional pharmaceutical therapy towards natural-based therapies utilizing medicinal plants. Plant-derived exosomes are thought to provide many therapeutic benefits for patients. Anti-inflammatory, antioxidant, wound healing, anti-cancer, hepatoprotective, anti-tumour, anti-obesity, anti-microbial and restorative properties have been established to be elicited from plant-derived exosomes. A deeper understanding of exosomes has the potential to introduce a new paradigm for natural medicine that makes use of substances which are more widely accessible, efficient and efficacious, as well as having a substantially lower number of adverse effects. Mammalian-derived exosomes stand to be a potential new approach in diagnostic biomarkers and therapeutic vehicle carriers. It was found that most exosome research is still carried out *In vitro*. This creates more possibilities for thorough investigations, particularly on exosome bioactivity, isolation, handling, and standardized mass production. In this research project, exosomes derived from *Lobostemon fruticosus* were analysed to gauge its wound healing potential. Nanotechnology is revolutionising many different aspects of drug delivery, offering a plethora of advantages. Further, it has an important therapeutic role to play going forward in improving the quality of life for all. Thus, incorporating traditional medicine into nanotechnology stands firmly as the future of pharmaceuticals.

Prior to being approved for usage in therapeutic settings, it is proposed that further in-depth research is required to completely comprehend exosomes derived from plants and mammals. Exosome-based clinical studies must be successfully conducted more frequently. Therefore, in addition to ongoing studies on exosomes, future research should concentrate on overcoming obstacles and restrictions associated with their application. Despite significant effort into this field of research, the understanding of exosomes is still limited by factors such as inefficient and inadequate separation methods, a lack of exclusive biomarkers and a lack of high-resolution visualisation techniques. Exosome analyses have rapidly advanced in the last decade, however the exact mechanisms of their biogenesis remains unclear. To fully grasp and analyse the exosomes' cargo contents and functions, which would in turn shed light on the biogenesis, advancements in exosome separation and purification methods are required. Once such limitations are overcome, opportunities to manipulate the composition of exosomes, its properties and cell interactions for the advancement of their therapeutic applications, will arise. In conclusion, to fully utilise the potential of exosomes, up-to-date research and new technologies need to be integrated. This will set the foundation for their therapeutic applications.

Chapter 3: Materials and Methodology

3.1 Materials

One batch of the plant sample of *Lobostemon fruticosus* was obtained from a local supplier. Reference substances of the potassium tablets, distilled water, deionized water, sucrose, dimethyl sulfoxide, Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), BSA, HaCaT cells, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide), dimethyl sulfoxide and 1% penicillin-streptomycin (P/S) were purchased from Sigma-Aldrich. The Pierce™ bicinchoninic acid (BCA) Protein Assay Kit, purchased from Thermo Fisher Scientific, was used to quantify the amount of protein in the exosomes. The kit contained a set of seven pre-diluted BSA standards.

3.2 Exosome isolation from dried and crushed *Lobostemon fruticosus* leaves

The sample of the *Lobostemon fruticosus* plant was received from a local supplier. The sample was made up of crushed, dried leaves. A commercial blender was used to pulverize 30 g of the sample after it had been powdered and combined with phosphate-buffered saline (PBS) in a 1:5 w/w ratio. The buffer solution was made by dissolving two potassium tablets in 150 mL of distilled water. The pH of the PBS was 7,4. Whilst, the approximate final concentrations of the solution was 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl. To remove impurities such as cell organelles, extracellular matrix and organic/inorganic compounds, the resulting mixture was purified by serial ultracentrifugation using the EBA 280 Tabletop Centrifuge (Hettich Instruments, United States of America (USA)). Ultracentrifugation is a commonly used method of purification and separation of low-molecular weight polymers using a centrifugal force created by rapid rotation [142]. Samples are rotated at exceptionally high speeds and depending on their respective masses, will settle at different speeds [142]. Stepwise centrifugation of the mixture was performed at 1000 x g for 10 minutes, 2000 x g for 20 minutes, 3000 x g for 30 minutes, and 10 000 x g for 60 minutes. At each stage, the supernatant was collected. The final sample was filtered through a 0.9 µm, 0.45 µm and 0.22 µm filter (Millipore, Billerica, MA, USA) before being centrifuged at 10 000 x g for 60 minutes. The samples were divided into two batches prior to lyophilization. Batch 1 was the original, plain sample. Batch 2 was the sample containing 10% w/w sucrose. Sucrose, a non-reducing disaccharide, was used as a lyoprotectant. Lyoprotectants function to protect various biomolecules against the stresses caused during lyophilization [143]. The exosomes were lyophilized utilizing the Freezone 12 freeze drier (Labconco, Kansas City, USA), for 12 hours at -80°C. The product was then stored at -70°C for further use.

3.3 Protein quantification

Following the manufacturer's instructions, the Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) was used to quantify the amount of protein in the exosomes. For the measurement of protein concentration, this kit provides a high-precision, detergent-compatible protein assay. The BCA protein assay combines protein-mediated Cu^{2+} to Cu^{1+}reduction in an alkaline medium with very accurate and focused colorimetric detection of the cuprous cation Cu^{1+} by BCA [144].

The reaction, which is often referred to as the biuret's reaction, begins with the chelation of copper with protein in an alkaline environment to create a light blue complex [144]. The kit contained a set of seven pre-diluted BSA standards. The peptides that include three or more amino acid residues combine with cupric ions to produce a coloured chelate complex in an alkaline solution of sodium potassium tartrate. The resulting reduced cuprous cations then reacts with BCA [144]. Chelation of two molecules of BCA with one cuprous ion yields the bright purple reaction product. With increasing protein concentrations, the water-soluble BCA/copper complex displays a potent linear absorbance at 562 nm. Compared to the light blue colour of the initial reaction, the complex is around 100 times more sensitive. Cysteine, cystine, tyrosine, and tryptophan, four of the protein's amino acid residues, have a significant impact on the process [144].

The kit contained a set of seven pre-diluted BSA standards. A series of dilutions of known concentrations are prepared from the protein and are assayed alongside the unknowns prior to the concentration of each unknown being determined based on the standard curve. The test tube procedure was followed for this assay. A volume of 0.1 mL protein sample was required to follow this procedure. Three samples of *Lobostemon fruticosus* exosomes were protein quantified. A control sample/blank was used – vial I. This contained 400 µL of diluent, added to 0 µL of stock (standard reference Albumin). This resulted in a final BSA concentration of 0 µL /mL. Vial A contained 0 µL of diluent, added to 300 µL of stock (standard reference Albumin). This resulted in a final BSA concentration of 2000 µL /mL. Vial B contained 125 µL of diluent, added to 375 µL of stock. This resulted in a final BSA concentration of 1500 µL /mL. Vial C contained 325 µL of diluent, added to 325 µL of stock. This resulted in a final BSA concentration of 1000 µL /mL.

0.1 mL of each standard and unknown sample were pipetted in appropriately labelled test tubes. 2 mL of the BCA working reagent was added into each of the test tubes. The test tubes were incubated at 37°C for 30 minutes. The samples in the test tubes were then analysed using the spectrophotometer set at 562 nm for 10 minutes. The standard curve was plotted

using the average blank 562 nm measurement versus the concentration in µg/mL. The standard curve determined the protein concentration of each of the unknown samples.

3.4 Scanning electron microscopy (SEM)

The SEM is a particular kind of electron microscope that concentrates an electron stream to create a range of signals on the surface of solid specimens [145]. It is a technique used for the visualization and characterization of surfaces and can be used to obtain information about the surface's topography and composition [145]. The lyophilized material was applied to a sticky carbon tape that was fastened to a metal stub. Two coats of Gold/Palladium were applied to the sample, which was then examined using the LEO 1550 FEG-SEM (1-30kV FEG-SEM, Electron Dispersive X-ray Spectroscopy, Electron Back-Scatter Diffraction).

3.5 *In vitro* scratch assay

An approach used to evaluate the role of molecular and cellular pathways in cell migration is the *In vitro* scratch assay. Additionally, the assay can be used to assess a compound's therapeutic potential prior to use in a clinical setting [146].

The *In vitro* scratch assay was carried out using HaCaT cells. The growth medium, composed of Dulbecco's modified eagle's medium (DMEM) containing 10% FBS and 1% penicillin-streptomycin (P/S), was seeded with HaCaT cells in a 96-well sterile culture plate at 4 x 10^3 cells/well. A 96-well sterile culture plate can hold approximately 180 – 200 µL cell suspension per well. Thus, a certain concentration and volume of cells is first prepared and then will the cells be seeded. In this case, 1 mL of the HaCaT cells were added to a total of 15 mL of the growth media. At 37°C under 5% CO_2, the cells were cultured for 2 consecutive days. Using a pipette tip, horizontal scratches were made on the cells. By washing with PBS, the unattached cells or debris were eliminated. All of the old media was pipetted out and replaced with 5 mL of PBS to "wash" the culture plate before replacing with the new cell media. The 5ml of PBS was then removed and the culture plate was replenished with 15 mL of new cell media. Exosomes from *Lobostemon fruticosus* (10^8 particles/mL), using a minute concentration of 200µg/mL, diluted in DMEM with 5% FBS and 1% P/S were used to treat the cells. Image J software was used to quantify the scratched area after photographs were taken at 0, 24, and 48 hours post the scratch creation. Based on the scratch size that was created at 0 hours, the respective percentage of wound closures were calculated. One concentration was used. A positive control, nor an untreated control was used. Results from this study were visually compared to a positive control (untreated cells).

3.6 ZetaSizer

Particle and molecular sizes are determined using ZetaSizer systems. This method measures the diffusion of particles moving with Brownian motion, and then uses the Stokes-Einstein relationship to translate this information to a size and a size distribution [147]. Targeted sizes of 100, 150, and 200 nm were prepared using optimized 2.0 mM nano-emulsions that contained 0.2 mg of sample diluted in 2 mL of distilled water. Using ZetaSizer (Nano ZS90, Malvern Instruments, Worcestershire, UK), the sample's polydispersity index (PDI) and average particle size were calculated. The sample was loaded into a plastic Zeta potential cuvette, after being diluted with deionized water 1000-fold. Prior to observing measurements, the temperature of the cell area was stabilized by equilibration for 2 minutes at room temperature (25 ± 1°C). Three experiments were run at three different temperatures. Three different temperatures were used to average the results as temperature has an influence on the ZetaSizer results.

3.7 Energy-dispersive X-ray spectroscopy

Energy-dispersive X-ray spectroscopy is an analytical technique used to identify and quantify the elemental composition of materials. This non-destructive method is commonly used alongside the SEM [148]. The process involves focusing an electron beam on a sample, and this yields the emission of characteristic X-rays from the atoms contained within the sample. Each element in the sample emits X-rays at specific energy levels, which are unique to that element. By detecting the emitted X-rays, energy-dispersive X-ray spectroscopy can determine the elements within the sample upon analysis [148].

For this study, this test was performed using externally using the following protocol. 1 mm^3 of the sample was added to 4% paraformaldehyde and 2% osmium tetroxide [148]. The sample was proceeded to be washed with 0.1 M phosphate buffer before being dehydrated by a series of incubations in 30%, 50%, 70% ethanol [148]. Dehydration continues by incubation steps in 95% ethanol, absolute ethanol and propylene oxide, after which samples are embedded in epoxidic resin. To prevent movement of elements, specimens are maintained at low temperature until they are stabilized by freeze-drying. A standard was made by dissolving known concentrations of salts in a 20% albumin solution. The droplets of the solution were then frozen. Upon which, the droplets were prepared following the procedure as the sample [148]. For this study, a positive control was not used.

3.8 *In vitro* cell viability

A cell viability assay essentially counts the number of living cells in a sample, as viability is a function of cellular death and cell proliferation [149]. Cell viability assays are used to measure

the physical and physiological health of cells with regards to therapeutic treatments, chemicals or extracellular stimuli. The growth media, which consisted of Dulbecco's modified eagle's mixture (DMEM) with 10% FBS and 1% penicillin-streptomycin (P/S) and 4 x 10^3cells per well, was used to seed the HaCaT cells onto a 96-well culture plate at 4 x 10^3 cells/well. A 96-well sterile culture plate can hold approximately 180 – 200 µL cell suspension per well. Thus, a certain concentration and volume of cells is first prepared and then will the cells be seeded. In this case, 1 mL of the HaCaT cells were added to a total of 15 mL of the growth media. At 37°C under 5% CO_2, the cells were left to attach for 24 hours.

The cells were then treated for 24 and 48 hours with *Lobostemon fruticosus* exosomes (1, 5, and 10 x 10^8 particles/mL) after being rinsed with PBS. The concentration of exosomes used was 200 µg/mL. The cell viability was assessed using the MTT assay following treatment with *Lobostemon fruticosus* exosomes. The MTT solution was prepared using a 5 mg/mL solution in PBS. This solution was sonicated. 50 µL MTT reagent along with 50 µL media containing no sample was prepared as the control. 50 µL of sample and 50 µL of MTT solution was added into each well. This was then incubated at 37°C for 3 hours. After incubation, 150 µL of MTT solvent (4 mM HCl, 0.1% NP40 in isopropanol) was added into each well. The cell plate was wrapped in foil and shaken on an orbital shaker for 15 minutes. The formazan crystals that were formed were further dissolved in the dimethyl sulfoxide and using an ELISA plate reader, the absorbance was determined at 570 nm – the reference wavelength. The absorbance readings were compared with the untreated control sample.

3.9 Analysis of data

Descriptive statistics were used to analyse results obtained from the characterisation tests. A grounded theory approach was used to analyse the concepts, categories and themes that emerged from the results obtained in this study. Cell viability of the HaCaT cells treated with the Lobostemon fruticosus exosomes was tabulated. This allowed for a comparison between the treated cells, a positive control and a negative control. These results were also represented graphically.

The image J software was used to quantify the wound site of the HaCaT cells from the *In vitro* scratch assay. Based on the scratch size that was created, the software calculated the percentage of wound closure. It was also able to denote the mean size of the wound site. This assay was performed once, over a period of 48 hours.

The protein concentration of the exosomes was calculated from the BSA calibration curve by using the linear regression equation. The standard curve was plotted using the average blank

562 nm measurement versus the concentration in µg/mL. The standard curve determined the protein concentration of each of the unknown samples. This assay was performed once.

Using ZetaSizer, the sample's polydispersity index (PDI) and average particle size were calculated. This test was performed three times, over three different temperatures.

3.10 Ethics

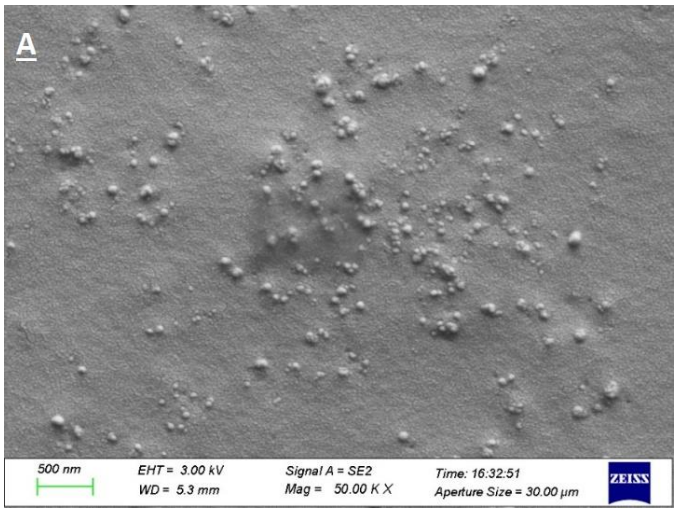
This research was conducted without the use of animal or human subjects. Hence, approval by the Wits Human Research Ethics Committee, nor the Animal Research Ethics Committee was required. This research was covered under current supervisor ethics.

Chapter 4: Results

4.1 Isolation and characterisation of *Lobostemon fruticosus exosomes*

By mechanically pulverizing and serial centrifuging the dried and crushed plant leaves, *Lobostemon fruticosus* exosomes were isolated. The exosomes were characterized using SEM, ZetaSizer, Energy-dispersive Xray spectroscopy (EDS elemental analysis), and the Pierce™ BCA Protein Assay Kit in accordance with the MISEV (minimum information for studies of extracellular vesicles) 2018, an ISEV (International Society for Extracellular Vesicles) recommended guideline.

The sizes of the exosomes, which ranged from 41 to 67 nm, were round and spherical (**Figure 4.1 and 4.2**). The elemental composition was established for the plain sample and for the sample containing 10% sucrose. Elements found in the plain sample included carbon, oxygen, with traces of palladium and gold (**Figure 4.3 and 4.4**, and **Table 4.1**). Elements found in the sample containing 10% w/w sucrose included chloride, potassium, with traces of oxygen, gold and sodium (**Figure 4.5 and 4.6**, and **Table 4.2**). The mean diameter was approximately 53.71 nm. The average particle size was determined by the ZetaSizer to be 166.2 d.nm (**Table 4.3**).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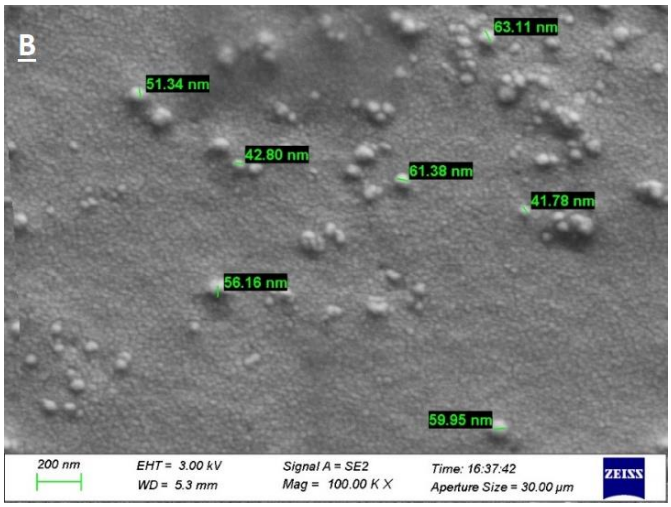
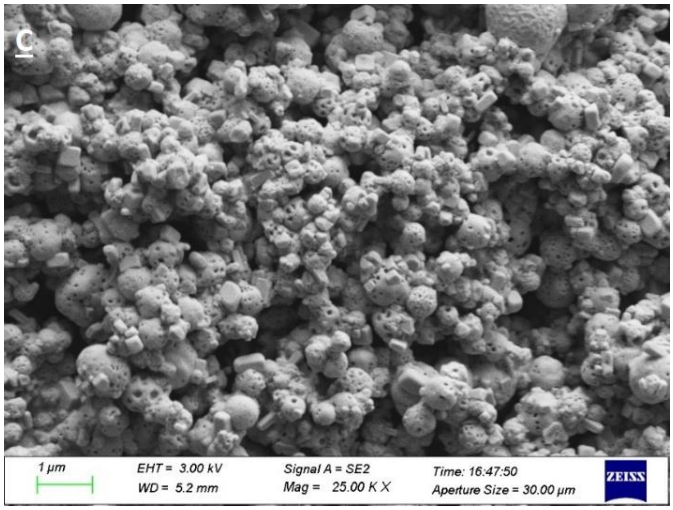
EHT = 3.00 kV
WD = 5.3 mm
Signal A = SE2
Mag = 50.00 K X
Time: 16:32:51
Aperture Size = 30.00 µm
ZEISS


B
63.11 nm
51.34 nm
42.80 nm
61.38 nm
41.78 nm
56.16 nm
59.95 nm
200 nm
EHT = 3.00 kV
WD = 5.3 mm
Signal A = SE2
Mag = 100.00 K X
Time: 16:37:42
Aperture Size = 30.00 µm
ZEISS


C
1 µm
EHT = 3.00 kV
WD = 5.2 mm
Signal A = SE2
Mag = 25.00 K X
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ZEISS

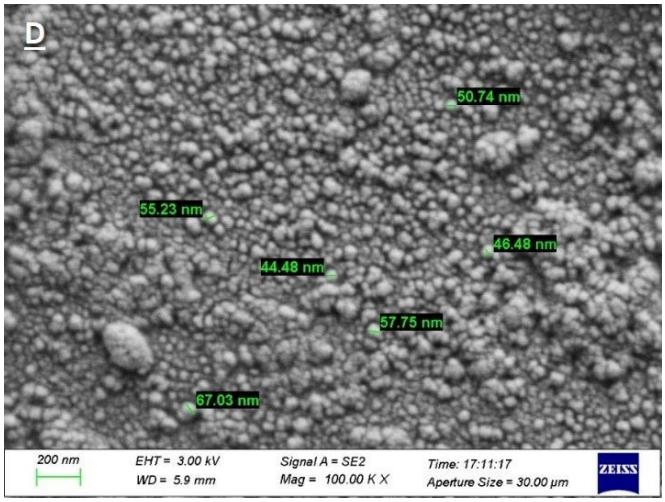
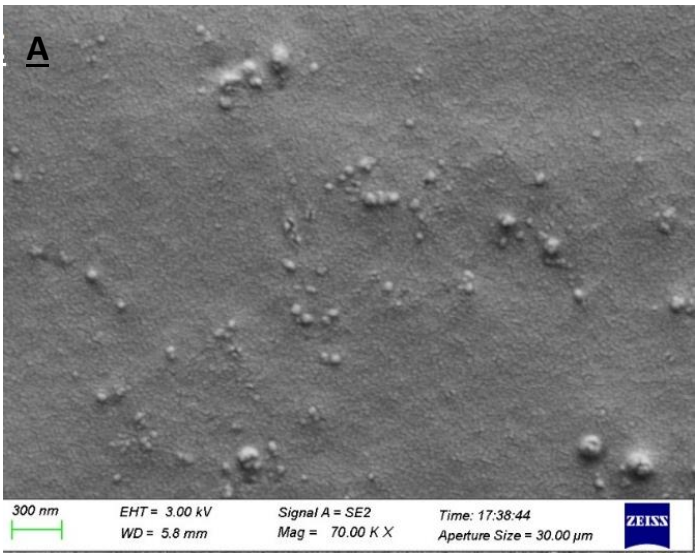


Figure 4.1: *Characterization of Lobostemon fruticosus nano-sprayed exosomes (A-D). Classic scanning electron microscope images of Lobostemon fruticosus nano-sprayed lyophilized exosomes of the plain sample. Figure A: Image magnified to 500 nm. Figure B: Image magnified to 200 nm, with individual exosome diameters. Figure C: Image magnified to 1 µm. Figure D: Image magnified to 200 nm, with individual exosome diameters.*



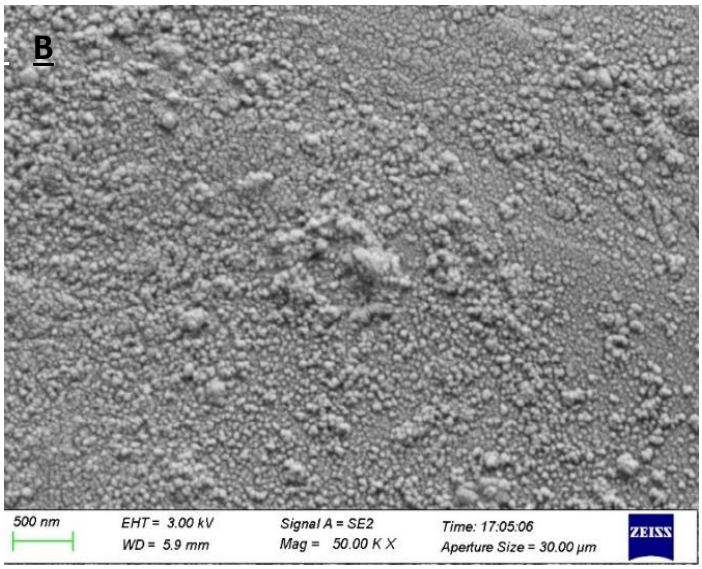


Figure 4.2: *Characterization of Lobostemon fruticosus lyophilized nano-sprayed exosomes containing 10% w/w sucrose (A-B). Classic scanning electron microscope images of Lobostemon fruticosus lyophilized exosomes of the sample containing 10% w/w sucrose. Figure A: Image magnified to 300 nm. Figure B: Image magnified to 500 nm.*

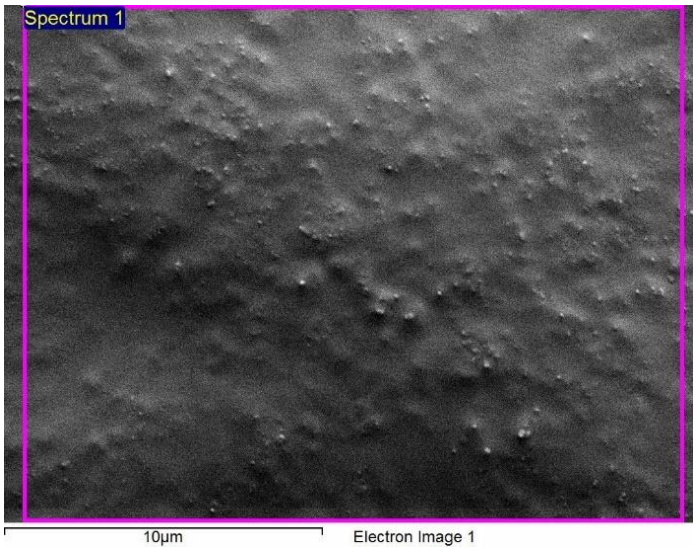


Figure 4.3: *Energy-dispersive spectroscopy image of the plain sample at a magnification of 10 µm.*

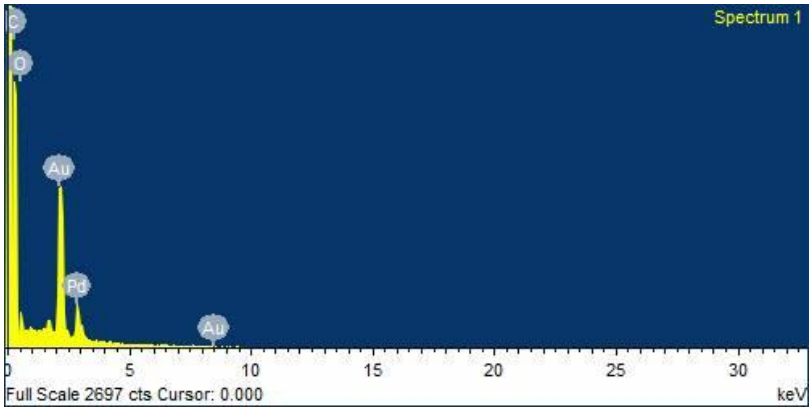


Figure 4.4: *Energy-dispersive spectroscopy graph of the plain sample illustrating elemental characterisation.*

Table 4.1 summarises the elemental data analysis taken from the energy dispersive spectroscopy of the plain sample.

Table 4.1: *Elemental data analysis summary of the plain sample.*

Element	Weight %	Atomic %
C K	48.40	85.28
O K	6.77	8.95
Pd L	10.36	2.06
Au M	34.47	3.70
Total	100.00	

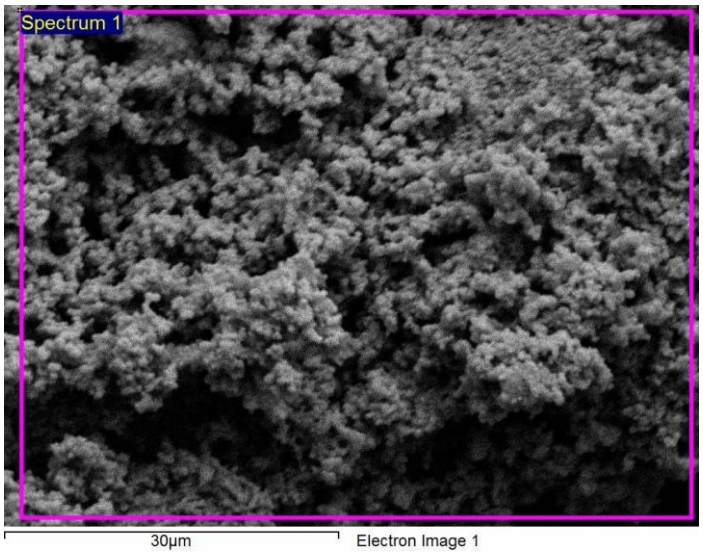


Figure 4.5: *Energy-dispersive spectroscopy image of the sample containing 10% w/w sucrose at a magnification of 30 µm.*

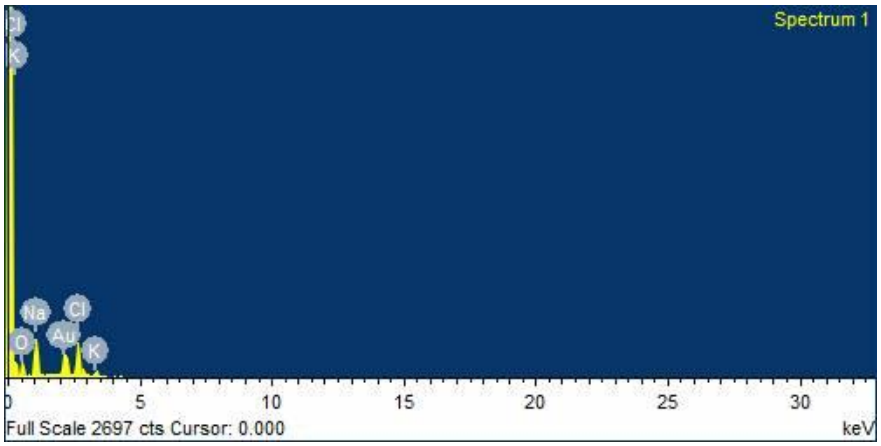


Figure 4.6: *Energy-dispersive spectroscopy graph of the sample containing 10% w/w sucrose illustrating elemental characterisation.*

Table 4.2 summarises the elemental data analysis taken from the energy dispersive spectroscopy of the sample containing 10% w/w sucrose.

Table 4.2: *Elemental data analysis summary of the sample containing 10% w/w sucrose.*

Element	Weight %	Atomic %
O K	15.56	36.05
Na K	12.31	19.85
Cl K	29.83	31.20
K K	6.50	6.16
Au M	35.81	6.74
Total	100.00	

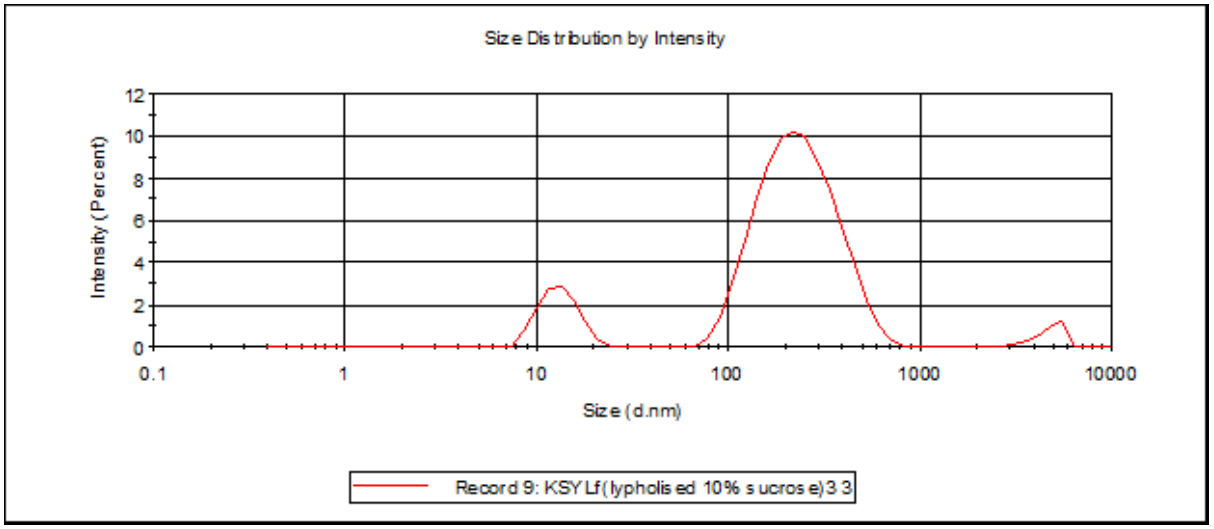


Figure 4.7: *Graph illustration of the size distribution by intensity from the ZetaSizer results.*

Table 4.3 summarises the results of the ZetaSizer.

Table 4.3: *Results of the ZetaSizer at 25°C for 140 seconds, with a count rate of 67.8 and an attenuation of 11.*

	Size (d.nm)	% intensity	Standard deviation
Peak 1	251.9	85.0	115.4
Peak 2	13.37	12.0	2.997
Peak 3	4882	2.9	684.4

According to the ZetaSizer results, the average particle size was 166.2 d.nm, with a PDI value of 0.258 and an intercept of 0.325.

Three samples of *Lobostemon fruticosus* exosomes were protein quantified. The results were as follows: The protein content for Sample 1 (plain) was determined to be 304.18 g/mL and had an absorbance value of 0.530 A. The protein content for Sample 2 (plain) was determined to be 305.94 g/mL and had an absorbance value of 0.533 A. The protein concentration in Sample 3 *(which contains 10% w/w sucrose*) was determined to be 2130.06 g/mL and had an absorbance value of 3.634 A. **Figure 4.8** depicts the standard calibration curve of the protein quantification using the Albumin standard (BSA) assay.

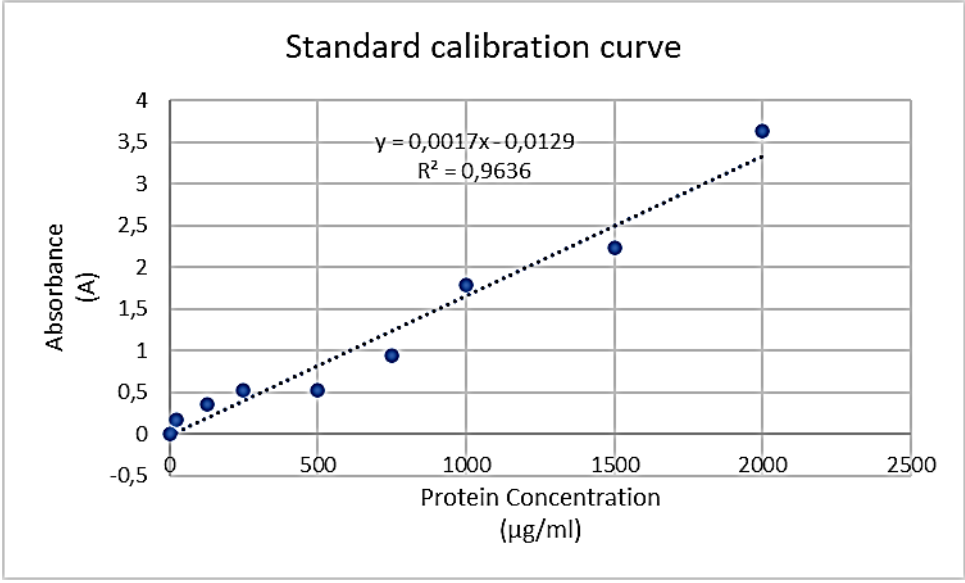


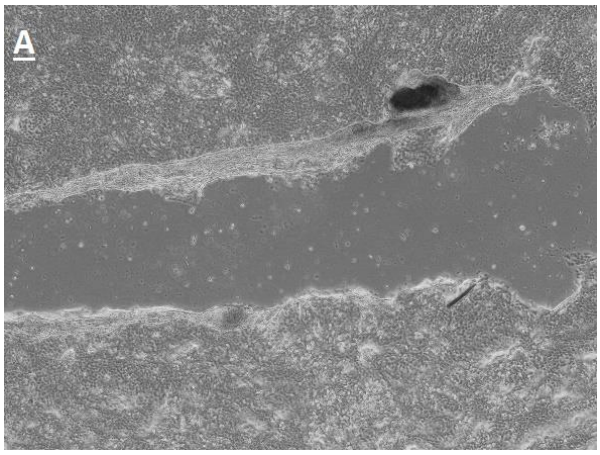
Figure 4.8: *Standard curve of BSA depicting the standard calibration curve of the protein quantification using the BSA assay. Each concentration was performed in a triplicate. Absorbance wavelength was 562 nm.*

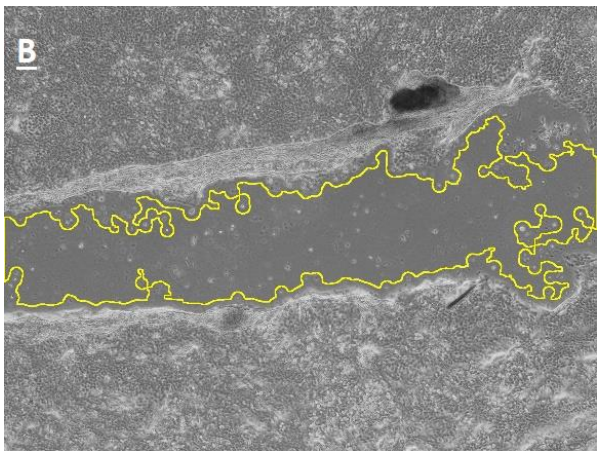
4.2 Effect of *Lobostemon fruticosus* exosomes on wound closure *In vitro* scratch wound model

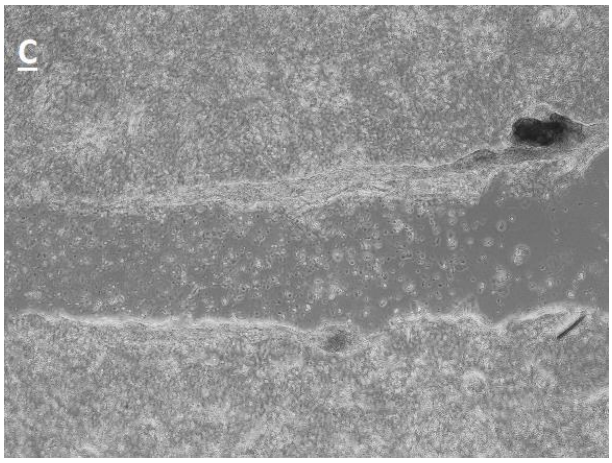
A critical stage in wound healing is the migration of skin cells to the wound site. HaCaT cells were scratched, followed by an incubation with *Lobostemon fruticosus* exosomes, to assess the effect of the exosomes on cell migration. In a time-dependent manner, scratch wounds on the HaCaT cells treated with *Lobostemon fruticosus* exosomes healed more rapidly. Results are tabulated and can be seen in **Table 4.4**. Using the Image J software, the area of the wound at 0 hours when the HaCaT cells were scratched was 644008 µm, with a mean value of 116.796 µm. Thus, the average size of the wounded area was 116.796 µm. Visually, the wound area was substantially reduced after 24 hours as a result of the cell migration to the wound site. The presence of two distinct wound sites suggested that the cells did not completely migrate to the wound site. Additional wound closure was seen at 48 hours. However, cell death and wounded areas persisted in five different locations. These findings combined suggest that *Lobostemon fruticosus* exosomes facilitate HaCaT cell migration.

Table 4.4: *The wound closure results.*

Time (hours)	Area 1 (µm)	Area 2 (µm)	Area 3 (µm)	Area 4 (µm)	Area 5 (µm)	Total area (µm)
0	644008	-	-	-	-	644008
24	12.966	1.217	-	-	-	14.183
48	22.941	3.342	1.265	2.975	1.528	32.051



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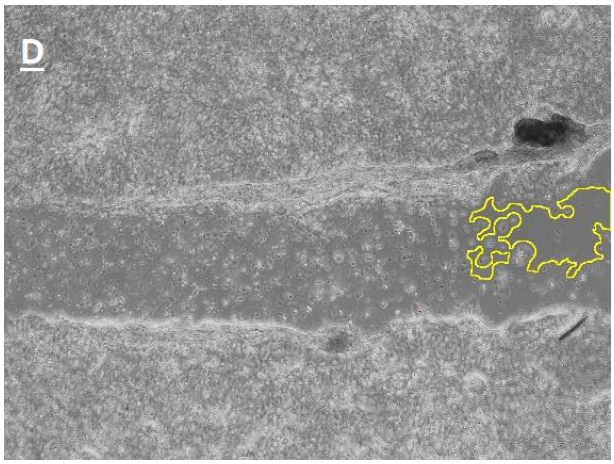
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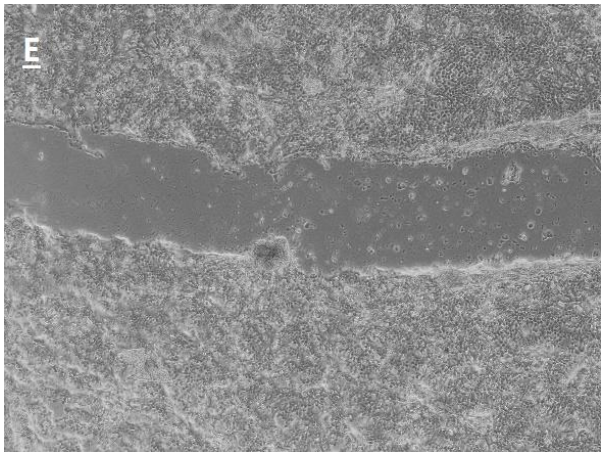
E

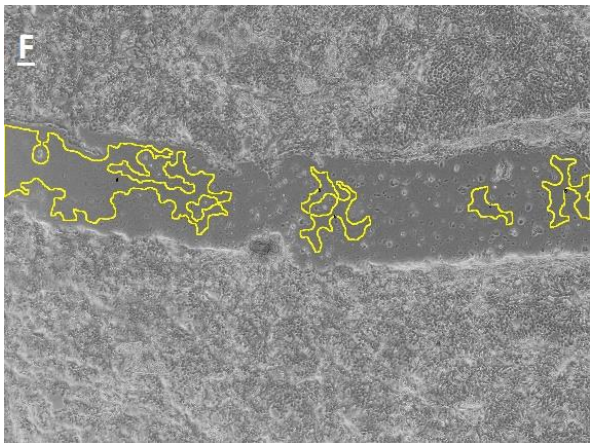

Figure 4.9: *Effect of Lobostemon fruticosus exosomes on the wound closure in the HaCaT cells. HaCaT cells in the scratched area as seen by optical microscopy (A-F). Neither poaitive nor negative controls were used as results were compared to previous research study results. After treatment with Lobostemon fruticosus exosomes, the images were captured at 0, 24, and 48 hours. Microscopy with a 10x magnification was used to capture the images. Using Image J, the wound closure area was established. A and B: captured after 0 hours: Area 1 – 644008 µm (mean 116.796 µm). C and D: captured after 24hours: Area 1 – 12.966 µm (mean 117.331 µm); Area 2 – 1.217 µm (mean 118.766 µm). E and F: captured after 48 hours: Area 1 – 22.941 µm (mean 122.824 µm); Area 2 – 3.342 µm (mean 111.690 µm); Area 3 – 1.265 µm (mean 117.329 µm); Area 4 – 2.975 µm (mean 114.969 µm); Area 5 – 1.528 µm (mean 111.259 µm).*

4.3 *In vitro* cell viability

The tetrazolium salt-containing MTT assay is a colorimetric assay that recognizes the synthesis of formazan, which turns the tetrazolium dye from yellow to purple in the presence of living cells with an active metabolism [149]. MTT is converted to formazan by the presence of NAD(P)H-dependent oxidoreductase enzymes in the viable cells. The MTT assay is used to measure cellular metabolic activity in terms of proliferation, cell viability and cytotoxicity [150]. **Table 4.5** summarises the absorbance values obtained from the positive controls, negative controls, and cells treated with various concentrations of exosomes. **Figure 4.10** below is a line graph illustration of the percentage relative cell viability of *Lobostemon fruticosus* exosomes plotted against a control variable.

Table 4.5: *The* absorbance values obtained from the positive controls, negative controls, and cells treated with various concentrations of *Lobostemon fruticosus* exosomes.

Abs positive control	Abs negative control	Treatment concentration (mg/mL)	*Cells treated with various concentrations of Lobostemon fruticosus exosomes*
1,41	0,05	4	2,661
1,415	0,039	2	2,192
1,42	0,069	1	1,783
1,422	0,063	0,5	1,684
1,482	0,086	0,25	1,665
1,49	0,074	0,125	1,901
1,508	0,077	0,0625	1,265
1,548	0,099	0,03125	1,331
1,461875	0,069625	-	-

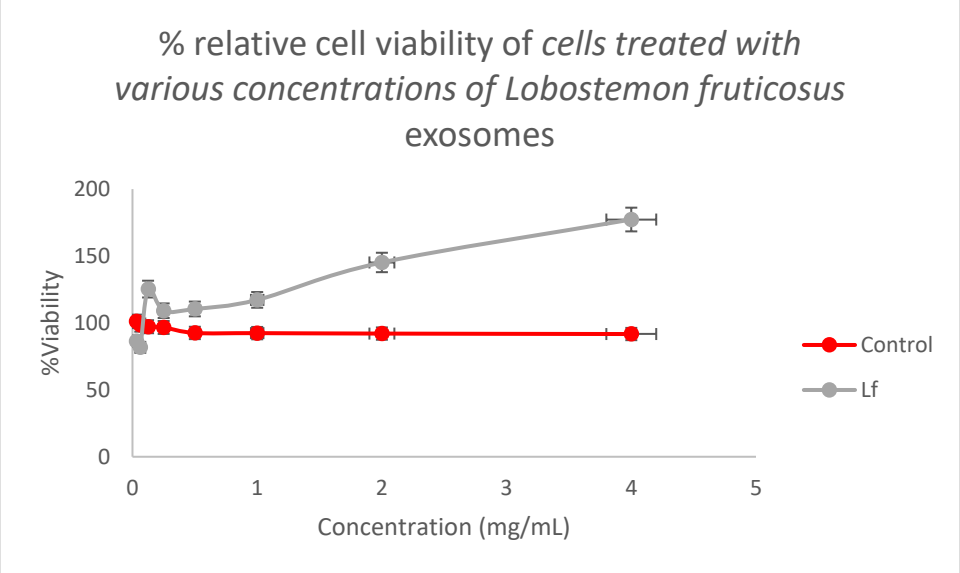

Figure 4.10: *Line graph illustration of the percentage relative cell viability of cells treated with various concentrations of Lobostemon fruticosus exosomes plotted against the positive control.*

*****LF – *Lobostemon fruticosus*

Chapter 5: Discussion

The skin serves as a biological barrier that protects against a variety of environmental threats including toxins [151], air pollution [152], UV radiation [151] and mechanical stress [153]. This harsh external environment often results in injury to the skin [154]. A wound is caused by internal and external breakdown of the epidermis and dermis, which disrupts normal anatomical structure and function. Complex physiological processes such as inflammation, cell proliferation and extracellular matrix remodelling all form part of normal wound healing [155]. Natural medicine has gained increasing popularity in the recent years for the treatment of a variety of human diseases [71]. It has been extensively proven that natural products have a variety of medicinal properties including antioxidant [154], anti-microbial [156] and anti-inflammatory activities amongst others [71]. Due to their diverse chemical backgrounds, substances derived from plants offer a foundation for the development of novel medications. As a result, reduced toxicity and chemical diversity are displayed with such products. Consequently, plant-based compounds are promising candidates for the development of novel drugs [4].

The scientific basis for traditional topical use of plants being used to treat wounds remains largely unexplored [157]. In this study, focus was placed on the wound healing effect of *Lobostemon fruticosus*. Plant remedies can possibly accelerate the wound healing process by acting in one or more of the wound healing phases. The migration of HaCaT cells at the wound site is a key indicator of wound healing. Thus, in this study, the *In vitro* scratch assay was used to analyse the effectiveness of exosomes derived from *Lobostemon fruticosus* in the wound healing process.

Based on ultracentrifugation, plant-derived exosomes were extracted from the dried and crushed *Lobostemon fruticosus* plant and characterised in accordance with ISEV guidelines. Characterisation tests done on the exosomes included protein quantification, ZetaSizer, Energy-dispersive Xray spectroscopy (EDS elemental analysis) and scanning electron microscopy. Further, *In vitro* cell viability testing was performed. By using an *In vitro* scratch assay, the wound healing ability of *Lobostemon fruticosus* exosomes were assessed. Should the results exhibit the exosomes promoting the migration of HaCaT cells to the wound site, i.e. accelerating the wound healing process, it can be suggested that *Lobostemon fruticosus* stands as a viable wound healing candidate.

Exosomes were isolated from *Lobostemon fruticosus* by ultracentrifugation. During ultracentrifugation, vesicles in *Lobostemon fruticosus* were separated based on their sizes, whilst other small subcellular organelles such as vacuoles were removed. The final

Lobostemon fruticosus exosomes had a translucent to faintly yellowish appearance. This was indicative of typical EV characteristics.

With sizes ranging from 41 to 67 nm, SEM images showed that the exosomes had the expected spherical rounded shape. The mean diameter was within the range of the anticipated nano-size EVs at 53.71 nm. The average particle size was measured to be 166.2 d.nm using the ZetaSizer. These results substantiated typical EV characteristics already established by previous literature and studies conducted [14].

The protein concentration of *Lobostemon fruticosus* exosomes from the BSA calibration curve was calculated using the linear regression equation. Two plain samples (samples 1 and 2) and one sample (sample 3) containing 10% sucrose were used and values were calculated accordingly, with the absorbance values measured against a wavelength of 562A. According to the Beer–Lambert law ($C = \frac{A}{\in L}$, where $C = concentration\ of\ the\ protein, A =$ absorbance of the sample, $\in = molar\ extinction\ coefficient\ and\ L =$ light pathlength), the concentration of a protein is directly proportional to its absorbance, at a defined wavelength and at a constant pathlength [158]. This implies a linear relationship existing between the protein concentration and absorbance as the higher the protein concentration, the higher its absorbance. Sample 1 had an estimated protein content of 304.18 µg/mL and an absorbance value of 0.530 A. Sample 2 had an estimated protein content of 305.94 µg/mL and an absorbance value of 0.533 A. Sample 3 had an estimated protein content of 2130.06 µg/mL and an absorbance value of 3.634 A. As established, the protein concentration values correlate to the respective absorbance values in terms of a linear relationship obeying the Beer-Lambert law. These results confirmed that the exosomes were rich in proteins. Proteins are high-molecular-mass molecules made up of chains of amino acids [159]. Due to their remarkable stability, amide bonds, also known as peptide bonds, are abundantly prevalent in proteins [160]. The amide functional group has a carbonyl carbon atom attached to a nitrogen atom. Proteins contain nitrogen and sulphur atoms in addition to carbon, hydrogen and oxygen atoms, and many also contain traces of other elements [159]. In this study, carbon and oxygen were identified as the main elements in the plain sample by energy-dispersive X-ray spectroscopy, with minor amounts of palladium and gold also present. In contrast, the sample containing 10% sucrose contained chloride, potassium, with traces of oxygen, gold and sodium. Such results further confirmed the presence of proteins in the exosomes.

Rapid wound closure with a functional and aesthetically pleasing scar is the main objective of wound care [161]. Should wound healing be accelerated as a result of *Lobostemon fruticosus* exosomes, the main objective of wound care would be fulfilled with the completion of this research project. As a natural physiological response to tissue damage, wound healing

involves a variety of cell types, cytokines, mediators, and the vascular system [162, 161]. Haemostasis, chemotaxis, and enhanced vascular permeability are characteristics of the inflammatory phase of wound healing, which serves to prevent further injury, close the wound, remove cellular debris and microorganisms, and promote cellular migration [162]. The granulation phase, re-epithelialization, and neovascularization are characteristics of the proliferative phase [162]. Finally, the maturation and remodelling phase is where the wound achieves maximum strength as it matures [162]. Several studies have reported that using exosomes derived from *Lobostemon fruticosus* assists in wound healing [95, 94, 93, 37]. In this study, we investigated the wound healing potential of exosomes derived from *Lobostemon fruticosus* by an *In vitro* scratch assay. A scratch assay entails the indirect analysis of the migration of cells to the wound site in the proliferation stage of wound healing [163]. A 'scratch' is created in a cell monolayer of the model, upon which, images at the beginning and at regular intervals during cell migration to close the scratch are captured. These images are then used for comparison to measure the rate of migration of the cells [164]. As can be seen from the comparison of the images, *Lobostemon fruticosus* exosome treatment significantly promoted the migration of HaCaT cells to the wound site, thus implying rapid wound closure. These results correlated with previous studies conducted. A relatively minute concentration of 200µg/mL of *Lobostemon fruticosus* exosome treatment was used. Images were taken at 0, 24, and 48 hours following the 'damage' to the HaCaT cells. Images collected at 0 and 24 hours show distinct cell movement, i.e. indicating wound healing. The wound site decreased by 643993.817 µm after 24 hours, indicating a significant level of cell migration. After 48 hours, there has been additional cell migration towards the wound site, as well as cell death. Cell death can possibly be attributed to human error or due to issues with sterilisation of the wound model or toxicity that may have developed with the product overtime. Challenges were encountered during the growth stage of the HaCaT cells. To successfully scratch the HaCaT cells, the cells require 2 days to fully grow. On the first attempt of growth, the HaCaT cells died within 24 hours of culturing. Reasons can be attributed to the extra sensitivity of the cells, human error such as over-handling of the cells or incorrect incubation of the cells. As a reasonably minute concentration of exosomes were used, the scratch wound was not expected to fully close. A positive control, untreated cells, was used. These untreated cells were contained in a normal growth medium (DMEM containing 5% FBS and 1% P/S). A visual comparison was done between the positive control and the cells with the *Lobostemon fruticosus* exosome treatment.

With regards to the cell viability of the HaCaT cells, non-cytotoxicity was exhibited when treated at various concentrations of *Lobostemon fruticosus* exosomes. As is depicted, the percentage of cell viability increases significantly as the concentration treatment of

Lobostemon fruticosus exosomes increases. This can be further substantiated with the MTT assay results, as well as the plotted line graph.

Although *Lobostemon fruticosus* exosomes have been shown to have a definite potential for wound healing, uncertainty still exists regarding which factors of the plant species constituents are responsible for the wound healing process. Hence, this issue could be further addressed in future studies. Additionally, further research should be focused on the possible adverse effects of therapeutically using *Lobostemon fruticosus*. For clinical application approval, a strong backing of scientific evidence, along with animal testing and pre-clinical and clinical trials are required. Currently, there exists an inadequate amount of research conducted on *Lobostemon fruticosus.* The mechanism of action of the plant species constituents, pharmacokinetics, toxicity and essentially the plant's ethnopharmacological properties need to thoroughly be established.

Chapter 6: Conclusion

In this study, the wound healing potential of exosomes derived from *Lobostemon fruticosus* was examined. Using an efficient method based on ultracentrifugation, exosomes with wound healing potential from *Lobostemon fruticosus* was effectively isolated. Using characterisation tests such as protein quantification, ZetaSizer, Energy-dispersive Xray spectroscopy and scanning electron microscopy, it was demonstrated that *Lobostemon fruticosus* exosomes exhibited characteristics commonly associated to EVs. Such characteristics included a high protein content and nano-size diameters. It was further demonstrated that the *Lobostemon fruticosus* exosomes are non-cytotoxic towards the HaCaT cells at various concentrations. Moreover, in respect to wound healing, it was demonstrated that *Lobostemon fruticosus* exosomes promoted the migration of HaCaT cells to the wound site. Therefore rapid wound closure, the main objective of wound care, was fulfilled.

Plant-derived exosomes have a number of proven therapeutic benefits, as was seen from results of this study. It should be noted that emphasis should be placed on furthering research on *Lobostemon fruticosus* with regards to its phytochemical and pharmacological properties, as well as the mechanisms of action of its constituents such as the EVs, prior to its approval for clinical application. Using the already established medicinal traits of *Lobostemon fruticosus*, further research should be focussed on developing these traits for novel drug discovery. Additionally, emerging research regarding the ability of plant-derived exosomes to serve as drug-delivery carriers is surfacing. Results reflecting from this study are speculative of this. Utilising their favourable biocompatibility, low toxicity and immunogenicity, ability to shield their contents from the external environment, target-specific ability, the ability to lengthen a drug's cycle and its duration of action, plant-derived exosomes can serve as novel drug delivery carriers. Further, the ability to be produced in high numbers and ability to penetrate the blood-brain barrier without passing through the placenta is advantageous in respect to drug delivery. The findings from this study supports literature and findings from previous studies conducted regarding the non-cytotoxicity and wound healing potential of exosomes derived from *Lobostemon fruticosus*. Further, as the characterisation test results from this study of the isolated exosomes displayed traits belonging to those of nanoparticles, novel drug discovery incorporating nanotechnology can be investigated. Therefore, it can be put forward of utilising *Lobostemon fruticosus* exosomes as a platform for drug delivery.

Despite the challenges faced and subsequent human errors experienced during this project, positive results were yielded. It can be concluded that the wound healing properties observed with the use of *Lobostemon fruticosus* can be attributable to the functions and properties of the exosomes that was derived from the plant species. Yet again, additional research needs

to be focused on its therapeutic efficacy and pharmacological action through animal studies. For the future development and discovery of pharmaceutical products and drugs from *Lobostemon fruticosus,* more in-depth research involving pre-clinical and clinical trials is required. Currently, there is a lack of scientific information regarding the pharmacokinetics and toxicity of this plant. Therefore, emphasis should be placed on continuing ethnopharmacological research on the plant species.

One of the limitations of this study was that only the scratch assay incorporating the *Lobostemon fruticosus* treatment was analysed. For future prospective studies, it can be suggested that whilst undertaking the scratch assay incorporating the *Lobostemon fruticosus* treatment, a concurrent scratch assay without any treatment should be conducted. A study incorporating a negative and positive control can also be suggested for more confirmative results. Such results will illustrate direct comparisons in terms of the rate of wound healing. Further, when culturing HaCaT cells, more caution needs to be placed on the proper incubation techniques and on the prevention of the over-handling of the cells to avoid cell death.

In conclusion, using an evidence-based ethnopharmacological approach, this study has provided a scientific basis for plants used traditionally in wound healing. As from the aforementioned, it is proposed that *Lobostemon fruticosus* exosomes have tremendous potential to be used as a drug delivery system in wound healing and skin regeneration.

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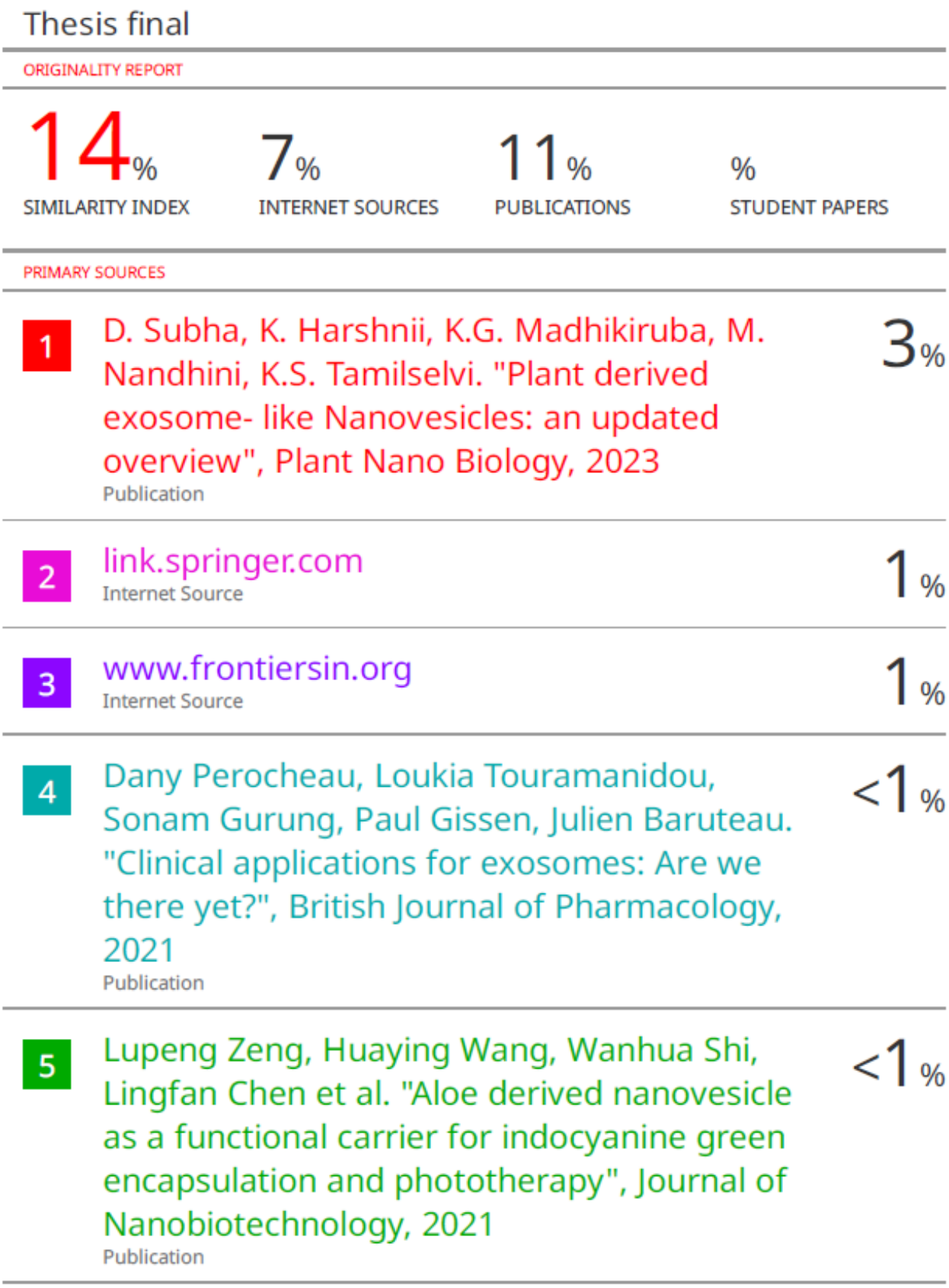
Appendix

Figure A1: *Turnitin Originality Report*



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