



**SYNTHESIS OF SILVER AND ZINC OXIDE NANOPARTICLES USING
STINGLESS BEE HONEY AND THEIR BIOCOMPATIBILITY IN WOUND
GAUZE APPLICATION**

By

NORFARINA BINTI BAHARI

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
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Philosophy**

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This study investigates the synthesis of silver nanoparticles (Ag-NPs), zinc oxide nanoparticles (ZnO-NPs), and bimetallic Ag/ZnO-NPs using stingless bee honey (SBH) as a natural reducing and stabilising agent. Bioactive compounds in SBH, including flavonoids, phenolics, and organic acids, facilitated nanoparticles (NPs) formation. Physicochemical characterisation showed average particle sizes of 30.09 ± 12.36 nm (Ag-NPs), 19.33 ± 4.21 nm (ZnO-NPs), and 33.05 ± 10.63 nm (bimetallic Ag/ZnO-NPs), with spherical morphology. Statistical analysis using one-way ANOVA followed by Tukey's post-hoc test confirmed significant differences ($p < 0.05$) in the antimicrobial efficacy of the NPs. Ag-NPs exhibited the highest antibacterial activity, with inhibition zones of 16.91 ± 0.40 mm against *Staphylococcus aureus* (*S. aureus*) and 17.43 ± 0.26 mm against *Escherichia coli* (*E. coli*), which were significantly greater than those of ZnO-NPs (13.50 ± 0.73 mm and 14.01 ± 0.95 mm) and bimetallic Ag/ZnO-NPs (13.02 ± 0.45 mm and 14.06 ± 0.54 mm).

Cytotoxicity assays revealed that Ag-NPs and ZnO-NPs were toxic to HaCaT cells at IC₅₀ values of 10 µg/ml and 85 µg/ml, respectively, whereas bimetallic Ag/ZnO-NPs showed no significant toxicity ($p > 0.05$). No cytotoxic effects were observed on 3T3 and MRC5 cells up to 100 µg/ml. In vivo wound healing analysis demonstrated that ZnO-NP-treated gauze significantly accelerated wound closure by day 4, achieving a closure rate of up to 51% ($p < 0.05$). Bimetallic Ag/ZnO-NPs showed consistent healing effects by day 8, with no statistical difference compared to ZnO-NPs at later stages ($p > 0.05$). Histopathological examination confirmed well-organised skin regeneration, including an intact dermis, keratinocyte proliferation, and adnexal structures, with minimal inflammation. Biochemical and hematological assessments showed no statistically significant differences among rats treated with synthesised NPs, commercial Durafiber Ag, and untreated gauze ($p > 0.05$). These findings highlight the potential of SBH's synthesised NPs as innovative wound healing agents, offering statistically significant antimicrobial properties, enhanced wound closure, and biocompatibility. The accelerated healing effects of ZnO-NPs and the sustained performance of bimetallic Ag/ZnO-NPs support their application for various wound types. This study underscores the need for further research to optimise formulations, explore novel biomaterials, and conduct clinical evaluations for practical translation into wound care applications.

Keywords: Antimicrobial, cytotoxicity, nanoparticle, stingless bee honey, wound healing.

SDG: GOAL 3: Good Health and Well-being & GOAL 9: Industry, Innovation & Infrastructure.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**SINTESIS NANOPARTIKEL PERAK DAN ZINK OKSIDA MENGGUNAKAN
MADU LEBAH KELULUT DAN KEBIOSERASIANNYA DALAM APLIKASI
KAIN PEMBALUT LUKA**

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Kajian ini menyelidik sintesis nanopartikel perak (Ag-NPs), nanopartikel zink oksida (ZnO-NPs), dan nanopartikel bimetalik Ag/ZnO-NPs menggunakan madu lebah kelulut (SBH) sebagai agen penurun dan penstabil semula jadi. Sebatian bioaktif dalam SBH, termasuk flavonoid, fenolik, dan asid organik, membantu dalam pembentukan nanopartikel (NPs). Pencirian fizikokimia menunjukkan saiz purata zarah ialah 30.09 ± 12.36 nm (Ag-NPs), 19.33 ± 4.21 nm (ZnO-NPs), dan 33.05 ± 10.63 nm (bimetalik Ag/ZnO-NPs), dengan morfologi sfera. Analisis statistik menggunakan ANOVA sehala diikuti ujian post-hoc Tukey mengesahkan perbezaan yang signifikan ($p < 0.05$) dalam keberkesanan antimikrob NPs. Ag-NPs menunjukkan aktiviti antibakteria tertinggi dengan zon perencatan 16.91 ± 0.40 mm terhadap *Staphylococcus aureus* (*S. aureus*) dan 17.43 ± 0.26 mm terhadap *Escherichia coli* (*E. coli*), yang secara signifikan lebih besar berbanding ZnO-NPs (13.50 ± 0.73 mm dan 14.01 ± 0.95 mm) serta bimetalik Ag/ZnO-NPs (13.02 ± 0.45 mm dan $14.06 \pm$

0.54 mm). Ujian sitotoksiti menunjukkan bahawa Ag-NPs dan ZnO-NPs adalah toksik kepada sel HaCaT pada nilai IC_{50} masing-masing 10 $\mu\text{g/ml}$ dan 85 $\mu\text{g/ml}$, manakala bimetalik Ag/ZnO-NPs tidak menunjukkan ketoksikan yang signifikan ($p > 0.05$). Tiada kesan sitotoksik diperhatikan pada sel 3T3 dan MRC5 sehingga kepekatan 100 $\mu\text{g/ml}$. Analisis penyembuhan luka secara *in vivo* menunjukkan kain kasa yang dirawat dengan ZnO-NPs mempercepatkan penutupan luka seawal hari ke-4 dengan kadar penutupan sehingga 51% ($p < 0.05$). Bimetalik Ag/ZnO-NPs pula menunjukkan kesan penyembuhan yang konsisten menjelang hari ke-8, tanpa perbezaan statistik yang ketara berbanding ZnO-NPs pada peringkat akhir ($p > 0.05$). Pemeriksaan histopatologi mengesahkan pertumbuhan semula kulit yang teratur, termasuk dermis yang utuh, percambahan keratinosit, dan struktur adnexal, dengan keradangan yang minimum. Penilaian biokimia dan hematologi menunjukkan tiada perbezaan statistik yang signifikan antara tikus yang dirawat dengan NPs yang disintesis, Durafiber Ag komersial, dan kain kasa tanpa rawatan ($p > 0.05$). Penemuan ini menekankan potensi NPs berasaskan SBH sebagai agen penyembuhan luka inovatif, menawarkan keberkesanan antimikrob yang signifikan secara statistik, penutupan luka yang lebih pantas, serta biokeserasian. Kesan penyembuhan yang dipercepatkan oleh ZnO-NPs dan keberkesanan berterusan bimetalik Ag/ZnO-NPs menyokong aplikasinya untuk pelbagai jenis luka. Kajian ini juga menyerlahkan keperluan untuk penyelidikan lanjut bagi mengoptimumkan formulasi, meneroka biomaterial baharu, serta menjalankan penilaian klinikal bagi menterjemahkan penemuan ini ke dalam aplikasi penjagaan luka yang praktikal.

Kata Kunci: Antimikrob, ketoksikan, madu lebah kelulut, nanopartikel, penyembuhan luka.

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LIST OF ABBREVIATIONS

SBH	stingless bee honey
NPs	nanoparticles
Ag-NPs	Silver nanoparticles
ZnO-NPs	Zinc oxide nanoparticles
Ag/ZnO-NPs	Silver/zinc oxide nanoparticles
Ag(NO ₃)	Silver nitrate
Zn(NO ₃) ₂	Zinc nitrate
LB	Luria-Bertani broth
DMSO	Dimethyl sulfoxide
ATCC	American Type Culture Collection
HaCaT	Human keratinocyte cell
MRC5	Human long fibroblast cell
3T3	Mouse embryonic fibroblast cell
CLSI	Clinical and Laboratory Standards Institute
CO ₂	Carbon dioxide
O-H	Oxygen – Hydrogen bond
C-OH	Carbon – Hydroxyl bond
C=C	Carbon – Carbon double bond
C-O	Carbon – Oxygen bond
C-N	Carbon – Nitrogen bond
N-H	Amine bond
Cl	Chlorine
Al	Aluminium
K	Potassium

Si	Silicone
O	Oxygen
Au	Gold
Ag	Silver
Zn	Zinc
LCMS	Liquid Chromatography Mass Spectroscopy
UV-vis	Ultraviolet-visible Spectroscopy
FESEM-EDX	Field Emission Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy
TEM	Transmission Electron Microscopy
FTIR	Fourier Transform Infrared Spectroscopy
XRD	X-ray Diffraction
UHPLC-Q-TOF/MS	Ultra-high-performance liquid chromatography quadrupole time of flight/mass spectrometry
UPM	Universiti Putra Malaysia
MARDI	Malaysian Agricultural Research and Development Institute
CANRES	UPM - MAKNA Cancer Research Laboratory
IBS	Institute of Bioscience, UPM
AMTREC	Animal Research Laboratory, MARDI
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
ZOI	Zone of inhibition
ROS	Reactive oxygen species
CAGR	Compound annual growth rate
λ	Wavelength
°C	Degree celcius

nm	Nanometre
g	Gram
mL	Mililitre
μL/mL	Microlitre per millilitre
cm	Centimetre
%	Percentage
v/v	Volume over volume
μL	Microlitre
min	Minutes
psi	Pounds per Square Inch
V	Volt
ppm	Part per million
M	Molarity
cm ⁻¹	Per centimetre
kV	Kilo volt

CHAPTER 1

INTRODUCTION

1.1 Overview

The global healthcare landscape faces significant challenges due to the prevalence of chronic wounds, which are characterised by their persistent and complex healing processes. These wounds, often caused by conditions such as diabetes, vascular diseases, and pressure ulcers, not only diminish patients' quality of life but also impose a considerable financial burden on healthcare systems. According to a recent report, the chronic wound care market was valued at USD 13.29 billion in 2023 and is projected to reach USD 24.03 billion by 2032, growing at a compound annual growth rate (CAGR) of 6.8% from 2024 to 2032 (N.d., 2024). This substantial growth underscores the urgent need for innovative therapies that address the increasing demand for chronic wound care products and services.

Conventional wound care approaches often fall short of meeting the multifaceted needs of chronic wounds, prompting researchers to explore new therapeutic modalities. Many studies on wound healing focus on assessing substances, methods, and mechanisms that expedite the healing process. However, identifying reliable outcome measures to robustly demonstrate the efficacy of these interventions remains a challenge. Bull et al. (2022) highlight that consensus among investigators regarding suitable endpoints for validating wound healing promotion is elusive. Effective endpoints must be precise, reproducible, and clinically significant to be valuable.

In recent years, there has been growing interest in natural and bio-based healing agents due to their therapeutic potential. Polverino et al. (2024) emphasise the increasing awareness and preference for natural solutions, which has shifted focus toward these alternatives in wound care. Among these, honey has gained significant attention due to its potent antimicrobial and anti-inflammatory properties, making it an excellent candidate for wound healing applications. Bonsignore et al. (2021) and Parvinzadeh Gashti et al. (2023) report that honey combats microbial infections, reduces inflammation, aids in debridement, and accelerates tissue regeneration. Furthermore, honey promotes key wound healing stages such as angiogenesis, epithelialisation, and granulation tissue formation (Nezhad-Mokhtari et al., 2021).

Similarly, stingless bee honey (SBH) is unique because it comes from diverse native vegetation, giving it a distinct sweetness with a hint of sourness and acidity. SBH has an acidic pH of 3.2–4.5, giving it a characteristic sour taste and aroma. It is more acidic than Apis honey due to its higher content of organic acids, esters, and inorganic ions such as phosphate, chloride, sulfate, and nitrate. The acidity of SBH is influenced by the nectar source, bee species, and enzymatic activity or bacterial communities. This acidic pH enhances its antibacterial properties by inhibiting the growth and survival of harmful microbes (Gadge et al., 2023). Unlike common honey, SBH typically contains higher water content, which supports the growth of beneficial probiotics. These probiotics secrete enzymes that, combined with those from the bees, enhance SBH's quality and therapeutic potential. Research indicates that SBH effectively combats microbial infections, reduces inflammation, aids in tissue

regeneration, and promotes wound healing stages like angiogenesis and epithelialisation (Nezhad-Mokhtari et al., 2021).

Building on these findings, SBH has emerged as a particularly promising therapeutic for wound management (Jalil et al., 2017). Sumarlin et al. (2021) and Zi Yun et al. (2021) describe SBH's robust antimicrobial and anti-inflammatory effects, which make it superior to conventional honey. Its unique storage method in cerumen pots enhances its therapeutic properties. According to Jalil et al. (2017), the transformation of nectar into SBH involves three distinct stages. The first stage is a physical transformation, during which a significant portion of the nectar's water evaporates. The second stage is biological, involving bacterial and yeast fermentation facilitated by microorganisms from the bee's environment. Finally, the third stage is a chemical transformation, during which enzymes secreted by the bees hydrolyse nectar sucrose into fructose and glucose.

Parallel to the growing interest in natural therapies, advancements in nanotechnology have opened new avenues for wound care. Nanoparticles (NPs) have shown superior properties compared to traditional wound dressings. Kalashnikova et al. (2015) categorise NPs into metal, metal oxide, and polymeric forms, each contributing to various stages of wound healing. Metal and metal oxide NPs, such as silver, zinc oxide, and titanium dioxide, exhibit strong antimicrobial properties and function as nanocarriers (Barroso et al., 2020; Bernal-Chávez et al., 2019). Similarly, polymeric NPs, including chitosan and hyaluronic acid, offer advantages such as biocompatibility,

biodegradability, and drug delivery potential (Graça et al., 2020; Moeini et al., 2020).

The mechanisms by which NPs aid wound healing are diverse. They include antimicrobial activity through membrane disruption, reactive oxygen species (ROS) generation, and enzyme inhibition. Additionally, they promote cell proliferation, angiogenesis, and collagen synthesis, as described by Homaeigohar and Boccaccini (2020). These properties make NPs ideal candidates for integration into modern wound dressings such as films, foams, hydrogels, and multilayer dressings, as noted by Ezhilarasu et al. (2020) and Stoica et al. (2020a).

In alignment with these advancements, this thesis aims to harness the therapeutic benefits of SBH in synthesising and characterising silver nanoparticles (Ag-NPs) and zinc oxide nanoparticles (ZnO-NPs). The combination of SBH's natural healing properties with the advanced functionality of NPs offers a revolutionary approach to wound care. Mendes et al. (2022) assert that metal-based NPs, such as those synthesised in this study, are ideal for wound bed applications due to their superior therapeutic efficacy. By integrating SBH and NPs into wound dressings, this research seeks to develop an innovative material that enhances healing outcomes and addresses the growing demand for effective wound care solutions.

1.2 Problem Statements

Chronic wounds are a significant healthcare challenge due to delayed healing, increased infection risks, and the limitations of existing treatments. Conventional wound dressings often lack sufficient antimicrobial activity, fail to accelerate healing, and are not biocompatible for extended use, particularly amidst rising antimicrobial resistance (Amirrah et al., 2020; Bayrami et al., 2020; Shafique et al., 2021; Tavakoli & Klar, 2020). This underscores the urgent need for advanced wound care solutions to address these complex issues.

Natural products, such as honey, have shown potential in wound management due to their antimicrobial and healing properties (Angioi et al., 2021; Croitoru et al., 2020). Among these, SBH stands out for its unique composition and broad-spectrum antimicrobial activity. However, its direct use in wound care is hindered by limitations, including variability in composition, poor spreadability, and contamination risks, which affect its therapeutic consistency and effectiveness.

Nanotechnology offers a promising solution to enhance SBH's efficacy by incorporating NPs like Ag-NPs and ZnO-NPs into wound dressings. These NPs exhibit enhanced antimicrobial properties, sustained release of therapeutic agents, and improved biocompatibility (Naskar & Kim, 2020). However, their potential cytotoxic effects, including oxidative stress and excessive inflammatory responses, pose challenges to their safe application

in wound healing (Contini et al., 2018; Czyżowska & Barbasz, 2022; Khanna et al., 2015; Wasef et al., 2020).

This research seeks to address these challenges by developing a bio-based wound dressing that combines SBH's natural healing properties with the technological advantages of Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs. The study focuses on synthesising and characterising these NPs using SBH to create a safe, effective, and sustainable wound care solution for chronic wounds, potentially transforming wound management practices.

1.3 Research Objectives

The main objective of this study is to evaluate the potential of Ag-NPs, ZnO-NPs, Ag/ZnO-NPs synthesised using SBH as bioactive agent impregnated in wound dressing. The specific objectives are:

- i. to assess the physicochemical properties of the synthesised NPs.
- ii. to assess the wound dressings impregnated with synthesised NPs.
- iii. to evaluate the antimicrobial activity and cytotoxicity of the synthesised NPs and wound dressings impregnated with synthesised NPs.
- iv. to evaluate the wound healing activity of the wound dressings impregnated with synthesised NPs.

1.4 Significance of the Study

This study represents a pivotal advancement in the field of wound care and nanotechnology, offering a novel approach that merges the ancient wisdom of natural remedies with cutting-edge scientific innovation. The integration of SBH with Ag-NPs and ZnO-NPs aims to create a groundbreaking wound dressing that addresses some of the most pressing challenges in chronic wound management.

One of the primary significances of this study lies in its potential to revolutionise the treatment of chronic wounds, which continue to be a major burden on healthcare systems worldwide. Chronic wounds, such as pressure ulcers, diabetic ulcers, and venous leg ulcers, are notoriously difficult to treat due to their prolonged healing times, high susceptibility to infections, and the frequent failure of conventional treatments to achieve complete healing. By developing a wound dressing that combines the natural antimicrobial and healing properties of SBH with the advanced capabilities of Ag-NPs and ZnO-NPs, this study could offer a more effective solution for promoting faster wound closure, reducing infection rates, and ultimately improving patient outcomes.

The use of nanotechnology in this study highlights the growing importance of nanoscale materials in medical applications. NPs, with their unique physicochemical properties, can interact with biological tissues in ways that traditional materials cannot. For instance, Ag-NPs and ZnO-NPs have demonstrated strong antimicrobial activity, even against antibiotic-resistant

bacteria, which is crucial for preventing infections in chronic wounds. Additionally, these NPs can promote wound healing by modulating inflammation, stimulating cell proliferation, and enhancing tissue regeneration. The ability to engineer NPs with specific sizes, shapes, and surface chemistries further allows for the customization of wound dressings to meet the unique needs of different wound types.

Beyond its immediate impact on wound care, this study also underscores the broader significance of integrating natural products with modern technology in medical practices. Honey has been used for centuries as a remedy for wounds, thanks to its antimicrobial, anti-inflammatory, and tissue-regenerative properties. However, its use in contemporary medicine has been limited by challenges such as variability in composition and potential contamination. By combining honey, specifically SBH with nanotechnology, this study not only enhances the efficacy and safety of honey-based treatments but also bridges the gap between traditional and modern medicine. This approach could lead to the development of new therapeutic modalities that harness the best of both worlds, providing patients with more effective and safer treatment options.

Furthermore, the successful development of this innovative wound dressing could have substantial economic implications. Chronic wounds are associated with significant healthcare costs, due to prolonged treatment durations, frequent hospitalisations, and the need for expensive interventions to manage complications like infections. A more effective wound dressing could reduce these costs by accelerating healing, minimising the need for additional

treatments, and reducing the incidence of wound-related complications. This could lead to a more efficient allocation of healthcare resources and better overall patient care.

The significance of this study also extends to the potential for future research and development in the field of biomedicine. The findings from this research could pave the way for further exploration of other natural products and nanomaterials in medical applications. For example, the success of SBH's synthesised NPs in wound care could inspire similar studies on other bioactive substances, such as plant extracts or other types of honey, in combination with different NPs. This could lead to the discovery of new therapeutic agents and the development of a wide range of innovative medical products.

1.5 Scope and Limitation of the Study

The scope of this study focuses on developing an innovative wound dressing material by leveraging the synergistic properties of SBH and SBH's synthesised Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs. SBH, known for its antimicrobial and wound healing properties, forms the foundation of the wound gauze. To enhance the antimicrobial effectiveness, SBH's synthesised Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs are integrated into the gauze. The study involves the synthesis of these NPs using a green synthesis method adapted from previous research by Maringgal et al. (2020) and Bayrami et al. (2020), which emphasises environmentally friendly processes.

Following synthesis, the SBH's synthesised NPs are incorporated into the wound gauze, with detailed experimental protocols ensuring optimal impregnation and uniform dispersion. Various characterisation techniques, including Scanning Electron Microscopy (SEM), X-ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), and UV-Vis Spectroscopy (UV-Vis), are employed to analyse the physical, chemical, and structural attributes of both the NPs and the wound dressing. These analyses provide crucial insights into the interactions between the NPs and the SBH matrix, which are essential for optimising the wound dressing's performance (Naskar & Kim, 2020).

The biological efficacy of the developed wound dressing is evaluated through in vitro and in vivo studies. In vitro studies assess the antimicrobial activity against common wound pathogens such as *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) (Angioi et al., 2021). In vivo studies, conducted using animal models, particularly rats, measure key outcomes like wound closure rates, bacterial load reduction, and histological changes in the wound tissue.

Despite its promising scope, the study has several limitations. One significant limitation is the variability in SBH composition, which can differ based on factors such as geographical origin, bee species, and available floral sources (Croitoru et al., 2020). The SBH used in this study is sourced from the *Heterotrigona Itama* (*H. Itama*) species and obtained from MARDI, Selangor. This variability can affect the antimicrobial and wound healing properties of the

honey, potentially impacting the study's outcomes (Rajendran et al., 2018). Moreover, Ng et al. (2020) reported that although the acidic environment in honey, attributed to gluconic acid, is considered an antibacterial factor, neutralising the acidity did not significantly reduce its antibacterial effect. They further noted that SBH with the lowest pH exhibited the strongest antibacterial activity, suggesting that its antibacterial effect may be influenced by acidity in combination with other factors.

Another limitation arises from the NPs synthesis method. The green synthesis method used, while environmentally friendly, may produce NPs with characteristics that differ from those created using other methods. Variations in size, shape, surface charge, and stability of the NPs could influence their biological efficacy, thus limiting the broader applicability of the study's findings (Contini et al., 2018). Additionally, the study's generalisability may be constrained by the types of wounds tested and the methods used to assess wound healing. The determination of wound margins, particularly in irregularly shaped wounds, can be subjective and prone to measurement errors, with wound contraction potentially leading to an underestimation of wound size (Andreu et al., 2015).

The use of animal models introduces further variability. Despite efforts to standardise experimental conditions, differences in genetics, age, health status, and behavior among the rats can influence their response to treatments, complicating the interpretation of the results (Shah & Amini-Nik, 2017). Moreover, the translation of these findings to human clinical settings

may presents additional challenges. Human wound healing is influenced by numerous factors, including underlying health conditions and immune responses, making it necessary to conduct further clinical trials to validate the efficacy of the SBH-based wound gauze in humans (Wasef et al., 2020).

1.6 Structure of the Thesis

This thesis is structured to provide a comprehensive exploration of the development and potential of SBH-based wound dressings enhanced with Ag-NPs and ZnO-NPs. Chapter 1 lays the foundation by offering an overview of the research topic, presenting problem statements that justify the need for the study, and clearly articulating its objectives. It also discusses the significance of the research, emphasising its potential impact on improving patient outcomes, reducing healthcare costs, and contributing to the broader field of nanotechnology in medicine. Additionally, the scope and limitations of the study are detailed, acknowledging the boundaries and challenges encountered. Chapter 2 provides an extensive review of existing literature, covering the current understanding of wound healing processes, the challenges posed by chronic wounds, and the role of SBH-based therapies in modern wound care. It also examines the synthesis methods for Ag-NPs and ZnO-NPs and their application in enhancing wound healing when combined with SBH, identifying gaps in knowledge that the current research aims to fill. Chapter 3 outlines the methodology, describing the synthesis of Ag-NPs and ZnO-NPs using SBH, and detailing the characterisation techniques employed to analyse their physicochemical properties. The chapter also covers the

incorporation of these NPs into wound dressings, ensuring uniform dispersion and effective integration. Chapter 4 presents the results and discussion, analysing the findings related to the physicochemical properties, antimicrobial efficacy, cytotoxicity, and in-vivo performance of the developed materials. The discussion interprets these results in the context of existing literature, highlighting innovations and challenges. Finally, Chapter 5 concludes the thesis by summarising the key contributions, reflecting on the translational potential of the developed wound dressing, and suggesting areas for future research, advocating for the advancement of natural product-based nanotechnology in medicine.

CHAPTER 2

LITERATURE REVIEW

2.1 Wound

A wound is defined as a breach in tissue integrity that impacts cutaneous, mucosal, or organ systems, disrupting the normal structure and function of tissues (Kida et al., 2020). This disruption can be caused by various forms of trauma, including mechanical (e.g., cuts and abrasions), thermal (e.g., burns), or chemical (e.g., corrosive substances). The nature of a wound is determined by its cause, depth, and the tissues involved, which can range from superficial skin layers to deeper organ structures.

Wounds are generally categorised into two types based on their healing process: acute and chronic (Figure 2.1). Acute wounds are those that result from sudden, traumatic injuries and typically follow a predictable healing process. Examples include lacerations, punctures, abrasions, and surgical incisions. These wounds usually progress through the standard phases of healing, including hemostasis, inflammation, proliferation, and remodelling within a timeframe of about two weeks in healthy individuals (Fallah et al., 2021). The rate and outcome of healing in acute wounds are influenced by factors such as wound size, depth, and the extent of tissue damage, with effective management leading to complete restoration of tissue integrity (Scepankova et al., 2021).

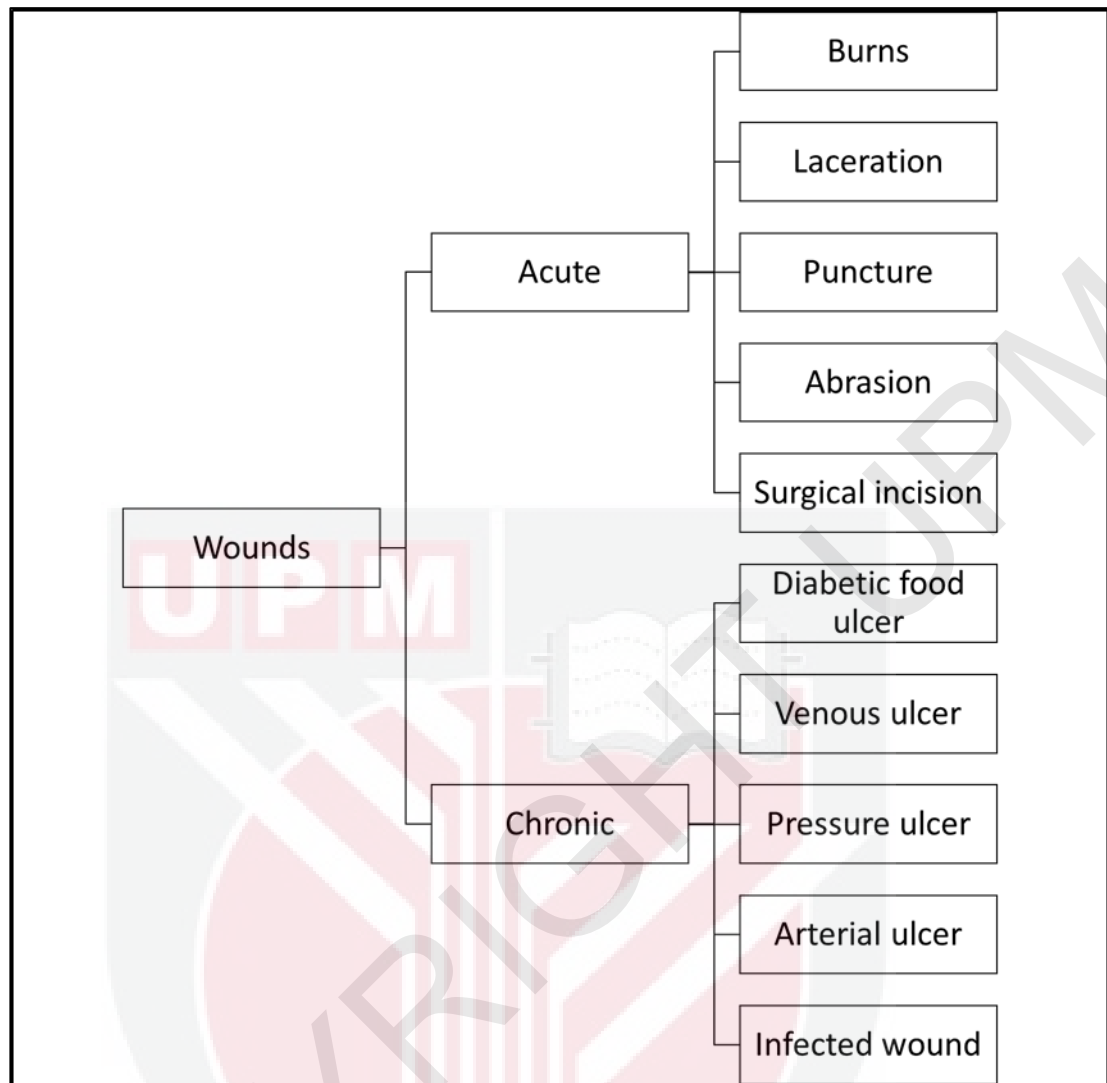


Figure 2.1: Types of wounds.

Chronic wounds, on the other hand, do not follow the normal healing trajectory and often require specialized intervention. These wounds include pressure ulcers, venous leg ulcers, diabetic foot ulcers, and chronic burns. They typically arise from underlying pathophysiological conditions that impair the skin's natural healing mechanisms, leading to prolonged damage and disruption of both the dermis and epidermis (Okur et al., 2020). Chronic wounds are frequently characterised by persistent bacterial colonisation, which can lead to complications such as antimicrobial resistance and biofilm

formation. This microbial environment impedes healing and can escalate into systemic infections like sepsis if left untreated (Scepankova et al., 2021). The microbial profile of chronic wounds evolves over time, initially dominated by gram-positive bacteria and later including a mixed population of gram-negative organisms (Lotfinia et al., 2023).

Several factors exacerbate the development and persistence of chronic ulcers, including aging, obesity, smoking, malnutrition, and immunosuppressive conditions such as AIDS or treatments like chemotherapy and radiotherapy (Okur et al., 2020). The depth and severity of the tissue injury also significantly impact the rate of wound healing, with deeper and more severe injuries typically leading to longer and more complex healing processes (Ashwini et al., 2023).

2.1.1 Wound Healing Process

Wound healing is a complex physiological process involving a series of orchestrated cellular and biomolecular mechanisms designed to repair damaged tissue and restore it to its pre-injury state (Tan et al., 2019). In general, the healing process can be broadly divided into two key mechanisms, which are wound contraction and epithelial growth. Wound contraction involves the retraction of the wound edges, while epithelial growth contributes to the formation of new skin tissue and helps to minimise further wound contraction (Kida et al., 2020).

The wound healing process is traditionally segmented into four distinct but overlapping phases which include, hemostasis, inflammation, proliferation, and remodeling (Figure 2.2).

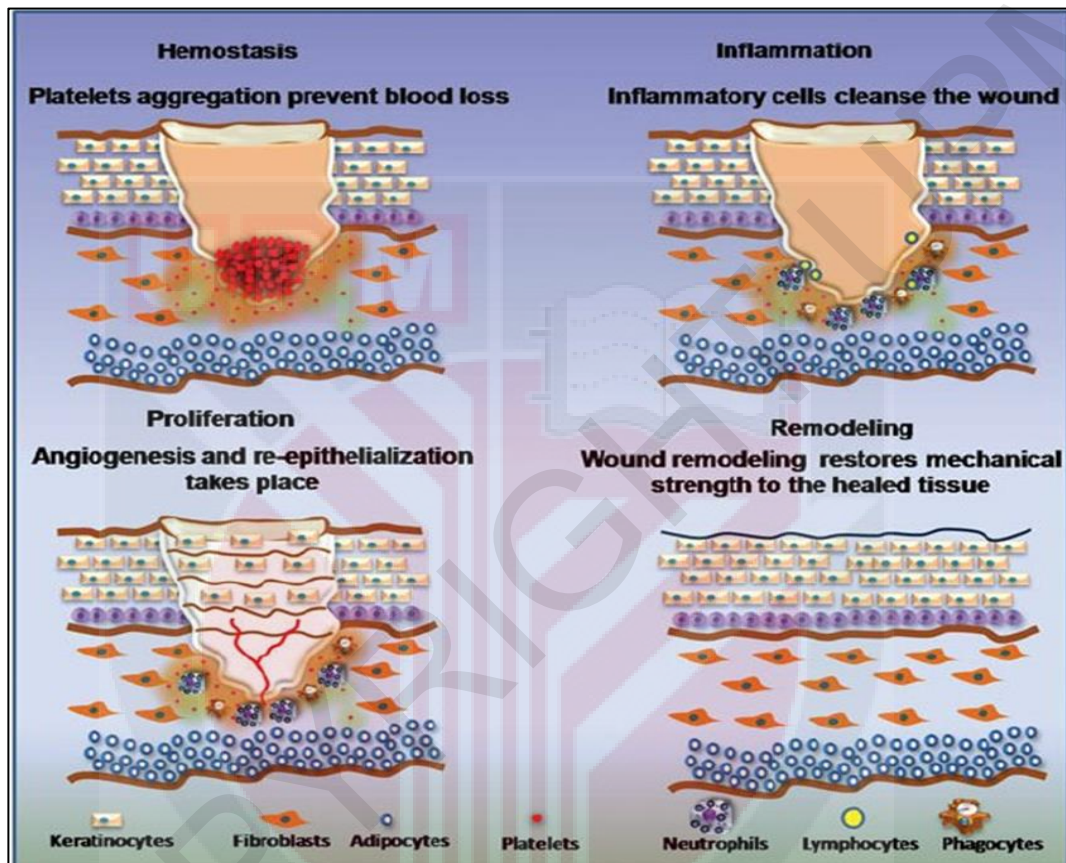


Figure 2.2: Wound healing phases
(Source: Sundaramurthi et al., 2014).

The initial phase, hemostasis, begins immediately after injury. Platelets aggregate at the wound site and form a thrombus, which serves to occlude the injured blood vessels and prevent excessive bleeding. This is accompanied by the formation of a fibrin mesh that stabilises the clot. Following hemostasis, the inflammatory phase begins, characterised by the infiltration of neutrophils and macrophages into the wound. These cells are crucial for removing bacteria, pathogens, and cellular debris through phagocytosis. This phase is

essential for preventing infection and setting the stage for tissue repair (Hamdan et al., 2017). The proliferation phase involves several processes aimed at tissue formation and repair. One of the key processes is angiogenesis, the formation of new blood vessels, which ensures an adequate supply of oxygen and nutrients to the healing tissue. Concurrently, granulation tissue forms to replace the damaged tissue, providing a foundation for new tissue development (Nur Eszaty et al., 2022). The final phase, remodeling, begins approximately three weeks post-injury and can continue for over a year. During this phase, the wound undergoes a transformation as collagen and other extracellular matrix (ECM) proteins are deposited, gradually restoring the structural integrity and functionality of the tissue.

Optimal wound healing requires the smooth progression through each of these phases. Disruptions at any stage, whether due to underlying conditions such as diabetes, age-related factors, ischemia, or external factors like infections, can significantly impair (Andreu et al., 2015; Blanco-Fernandez et al., 2021; Fallah et al., 2021; Nethi et al., 2019; Salimi & Mohammadipanah, 2021; Wang et al., 2020). Systemic comorbidities often lead to compromised wound perfusion, limiting the delivery of essential nutrients and oxygen, and hindering waste elimination. This disruption impairs the wound healing process by negatively affecting the tissue microenvironment, ultimately obstructing effective repair and regeneration (Dalisson & Barralet, 2019).

2.1.2 Wound Dressings

Recent advancements in wound dressing research have significantly enhanced our understanding of chronic wound aetiology and led to the development of more standardised and effective formulations. Wound dressings can be broadly categorised based on their composition and application, encompassing traditional, biomaterial-based, and synthetic types. Traditional dressings include gauze and cotton, which have been widely used due to their simplicity and availability. However, these materials often fall short in modern wound care due to their limited absorptive capacity and potential to adhere to the wound, particularly in burn management (Stoica et al., 2020a).

Biomaterial-based dressings include allografts, xenografts, and tissue derivatives, which leverage biological materials to enhance wound healing through natural processes. These materials are often biocompatible and can promote tissue regeneration, but their application may be limited by factors such as availability and risk of immunological reactions (Dhivya et al., 2015).

Synthetic dressings, which encompass films, membranes, foams, gels, composites, and sprays, offer a range of physical properties tailored to specific wound care needs. These materials are engineered to provide controlled moisture balance, enhanced exudate absorption, and protection against infection. Recent innovations in synthetic dressings focus on incorporating antimicrobial agents and improving biocompatibility to address the limitations of traditional materials (Polverino et al., 2024).

Recent research has highlighted several limitations in existing wound dressing technologies. Traditional cotton gauze, while commonly used, often fails to meet the demands of advanced wound care due to its insufficient absorptive capacity and tendency to adhere to wounds, which can impede healing and cause discomfort (Montaser et al., 2020). Consequently, there has been a concerted effort to enhance the functionality of textile-based dressings through modifications in fabric and fiber treatments. These modifications aim to improve wound healing rates and minimise adverse effects, such as those observed with conventional cotton gauze (Rehan et al., 2017).

Bio-based materials have gained significant attention as alternatives to synthetic and traditional dressings due to their inherent advantages, including biodegradability, biocompatibility, and bioactivity. Materials such as collagen, chitosan, cellulose, starch, and honey have been studied for their potential in wound care. For instance, collagen and chitosan have demonstrated antibacterial and cell-proliferative properties, while cellulose and starch contribute to moisture management and support tissue (Lotfinia et al., 2023; Mistry et al., 2021; Rezvani Ghomi et al., 2019; R. Singh et al., 2017; Zheng et al., 2020). Specifically, SBH has been noted for its antimicrobial properties and potential to accelerate wound healing, making it a valuable component in modern wound dressing innovations (Yupanqui Mieles et al., 2022). Figure 2.3 illustrates the types of wound dressing used for wound healing.

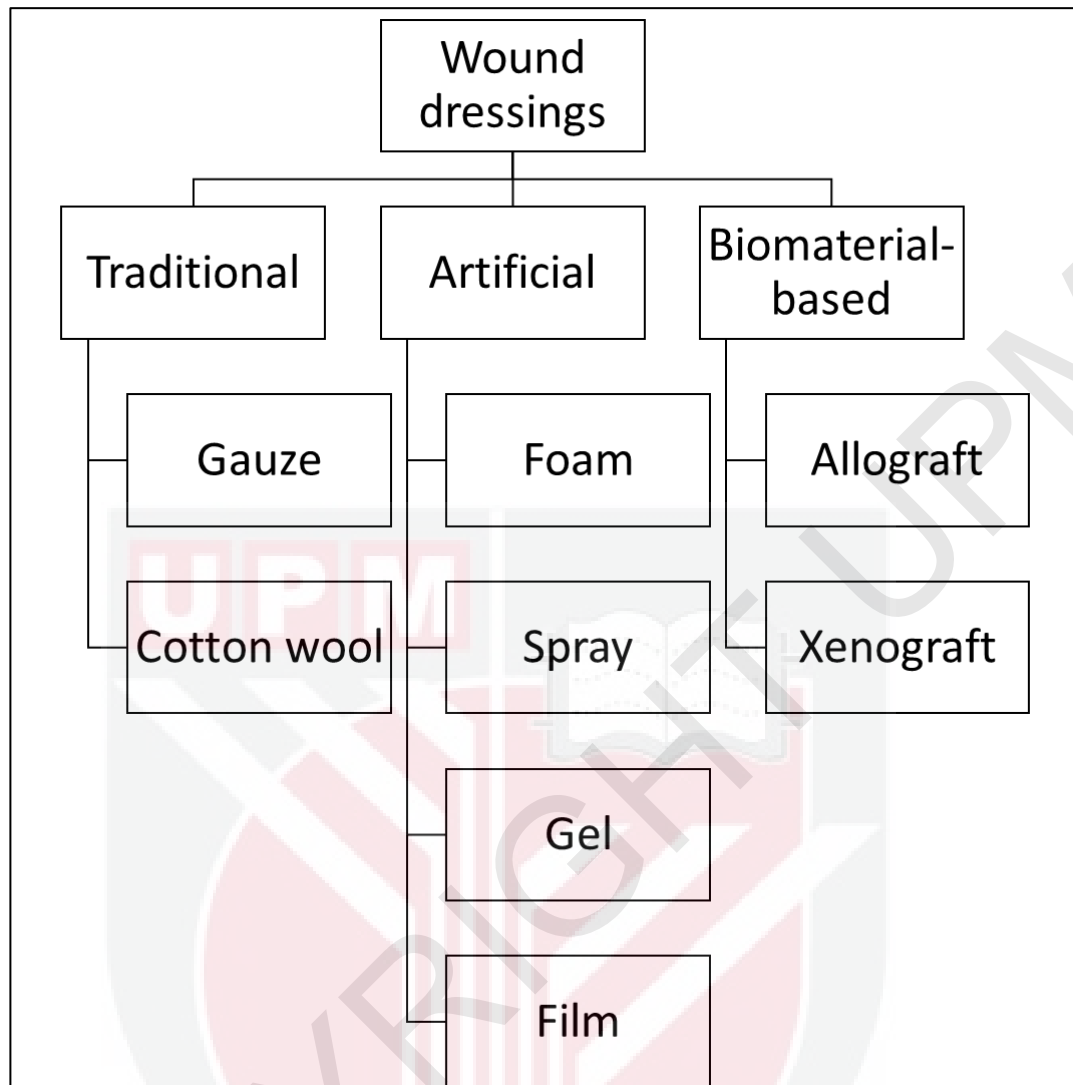


Figure 2.3: Types of wound dressings.

2.2 Stingless Bee Honey

SBH, produced by bees of the *Trigona* species, is a unique and valuable natural substance with distinctive physicochemical properties, market potential, and differences from common honey varieties like those produced by *Apis mellifera*. Known by various names across different regions, such as Meliponine honey, pot-honey, sugarbag honey in Australia, and Kelulut honey in Malaysia, SBH is gaining attention for its beneficial health properties and economic value (Fletcher et al., 2020).

One of the key factors that set SBH apart is its limited production capacity. Stingless bees typically produce only 1 to 5 kilograms of honey per colony annually, compared to the more prolific *Apis mellifera* bees, which can yield around 20 kilograms per hive each year (Martinello & Mutinelli, 2021). This lower production rate contributes to the higher market price of SBH, which can reach approximately \$100 per kilogram, significantly more than the \$20 to \$40 per kilogram price range of *Apis mellifera* honey (Hassan et al., 2023; Rozman et al., 2021). Additionally, according to Kanyanat et al. (2023), the selling price of SBH is approximately 10 times higher than that of honey from Thai-produced *Apis mellifera*. Despite its limited availability, the SBH industry is experiencing growth, particularly in Malaysia, where it is viewed as a promising superfood with substantial economic potential. In 2017, the farming area dedicated to stingless bees in Malaysia was approximately 1606.6 hectares, with expectations of continued expansion. Currently, SBH contributes around RM 200 million annually to the Malaysian economy, with projections suggesting this could rise to RM 350 million in the near future (Hassan et al., 2023).

SBH's physicochemical properties further distinguish it from common honey. It is characterised by higher levels of free acidity and moisture content compared to *Apis mellifera* honey, but it has lower pH and total soluble solids, which differ from established honey standards (Haron et al., 2022; Hassan et al., 2023; Majid et al., 2019; Nur Eszaty et al., 2022). The sugar content in SBH typically ranges between 60% and 75%, with extremes from 55% to 86%. This high sugar content creates an environment conducive to the growth of various

microorganisms, which is an important consideration in its handling and storage (de Paula et al., 2021). The physicochemical properties of SBH were summarised in Table 2.1.

In addition to its basic chemical composition, SBH is also rich in bioactive compounds that contribute to its health benefits. It contains significant levels of flavonoids, glucose oxidase catalase, and phenolic acids, all of which have been associated with positive effects on human health. These compounds are known for their antioxidant, antimicrobial, and anti-inflammatory properties, making SBH a valuable component in functional foods and natural medicine (Maringgal et al., 2019; Martinello & Mutinelli, 2021; Zamri et al., 2021; Zi Yun et al., 2021). The summary of bioactive compounds present in SBH were summarised in Table 2.2.

Table 2.1: Physicochemical properties for stingless bee honey.

SBH Types	Moisture (g/100g)	Total Reducing Sugar (g/100g)	Sucrose (g/100g)	Free Acidity (meq/100g)	pH	HMF (mg/kg)	Conductivity (mS/cm)	Ash (g/100g)	References
<i>Heterotrigona itama</i>	27.78 – 28.12	Nm	Nm	Nm	3.06 – 3.32	Nm	Nm	Nm	Kanyanat et al. (2023)
<i>Heterotrigona itama</i>	19.6 – 32.20	Nm	Nm	60.8 – 228.0	2.65 – 3.58	<LOD – 545.7	0.25 – 0.89	Nm	Hassan et al. (2023)
<i>Trigona apicalis</i>	35.2 – 35.9	Nm	Nm	263.3 – 269.5	2.96 – 3.57	<LOD – 133.2	0.67 – 1.40	Nm	Hassan et al. (2023)
<i>Geniotrigona thoracica</i>	31.9 – 32.1	Nm	Nm	156.5 – 200.2	3.02 – 3.20	<LOD – 55.9	0.54 – 0.65	Nm	Hassan et al. (2023)
<i>Geniotrigona thoracica</i>	28.17	Nm	Nm	Nm	3.36	Nm	Nm	Nm	Pimentel et al. (2022)
<i>Heterotrigona itama</i>	28.43	Nm	Nm	Nm	3.32	Nm	Nm	Nm	Pimentel et al. (2022)
<i>Tetrigona apicali</i>	42.00	13.00	Nm	Nm	3.20	0.26	Nm	1.40	Pimentel et al. (2022)
<i>Heterotrigona itama</i>	Nm	44.17 – 68.22	0.54 – 3.48	Nm	Nm	Nm	Nm	Nm	Haron et al. (2022)
Nm	21.52 – 31.00	24.21 – 72.9	<0.01 – 32.30	Nm	3.27 – 3.30	Nm	0.33 – 0.69	Nm	Kamal et al. (2021)

Table 2.1: Continued

SBH Types	Moisture (g/100g)	Total Reducing Sugar (g/100g)	Sucrose (g/100g)	Free Acidity (meq/100g)	pH	HMF (mg/kg)	Conductivity (mS/cm)	Ash (g/100g)	References
<i>Geniotrigona thoracica</i>	Nm	64.9	Nd – 1.23	Nm	Nm	Nm	Nm	Nm	Sujanto et al. (2021)
<i>Heterotrigona itama</i>	Nm	12.55 – 74.2	Nd – 2.09	Nm	Nm	Nm	Nm	Nm	Sujanto et al. (2021)
<i>Heterotrigona erythrogastra</i>	Nm	72.4	Nd	Nm	Nm	Nm	Nm	Nm	Sujanto et al. (2021)
<i>Heterotrigona bakeri</i>	30.87	Nm	Nm	Nm	3.17	Nm	0.57	0.29	Sharin et al. (2021)
<i>Geniotrigona thoracica</i>	31.61	Nm	Nm	Nm	3.10	Nm	0.75	0.24	Sharin et al. (2021)
<i>Trigona binghami</i>	41.54	Nm	Nm	Nm	2.71	Nm	2.15	1.11	Sharin et al. (2021)
<i>Heterotrigona itama</i>	27.00 – 31.00	Nm	Nm	Nm	Nm	Nm	Nm	0.15 – 0.90	Lim et al. (2019)
<i>Nm</i>	Nm	Nm	Nm	Nm	4.25 – 5.50	Nm	Nm	Nm	Maringgal et al. (2019)
<i>Heterotrigona itama</i>	23.33 – 31.00	Nm	Nm	Nm	3.17 – 3.68	Nm	0.04 – 0.22	0.14 – 0.89	Majid et al. (2019)
<i>Trigona sp.</i>	29.8 – 30.42	Nm	Nm	Nm	Nm	Nm	Nm	0.29 – 0.39	Ranneh et al. (2018)
<i>Trigona sp.</i>	25.00 – 31.00	54.90 – 87.00	0.31 – 1.26	Nm	3.15 – 4.66	8.80 – 69.00	0.49 – 8.77	0.01 – 0.12	Zulkhairi Amin et al. (2018)
Nm – Not mention.									

Table 2.2: Bioactive compounds of stingless bee honey

Bioactive compounds	References								
	Zhao et al. (2017)	Ranneh et al. (2018)	Zulkhairi Amin et al. (2018)	Biluca et al. (2020)	Chettri & Kumari (2020)	Hatamleh et al. (2020)	Silva et al. (2021)	Pimentel et al. (2022)	Rozman et al. (2022)
Gallic acid	✓	✓	✓			✓		✓	
Gallic acid hexose	✓								
Caffeoylquinic acid	✓								
Chlorogenic acid				✓		✓		✓	✓
Coumaric acid									✓
<i>p</i> -Coumaric acid		✓	✓	✓		✓		✓	✓
Cinnamic acid		✓	✓			✓		✓	
2-hydroxycinnamic acid		✓							
Salicylic acid				✓	✓	✓		✓	✓
Protocatechuic acid	✓			✓		✓		✓	✓
Ferulic acid				✓					✓
Trans ferulic acid						✓		✓	
Mandelic acid				✓		✓		✓	✓
Mandelic <i>p</i> -coumaric					✓				
Rosmarinic acid				✓		✓		✓	✓
Vanillic acid	✓			✓		✓		✓	✓

Table 2.2: Continued

Bioactive compounds	References								
	Zhao et al. (2017)	Ranneh et al. (2018)	Zulkhairi Amin et al. (2018)	Biluca et al. (2020)	Chettri & Kumari (2020)	Al-Hatamleh et al. (2020)	Silva et al. (2021)	Pimentel et al. (2022)	Rozman et al. (2022)
Vanillic hydroxymethyl					✓				
Isomer of vanillic acid	✓								
Caffeic acid	✓	✓	✓	✓		✓		✓	✓
Caffeic acid phenethyl ester		✓							
Caffeine	✓								
Ellagic acid									✓
Dihydrocaffeic acid									✓
Sinapic acid						✓		✓	✓
4-(hydroxymethyl) benzoic acid		✓							✓
4-aminobenzoic acid				✓					✓
Benzoic acid	✓					✓		✓	✓
Benzoic trans ferulic syringic chlorogenic					✓				

Table 2.2: Continued

Bioactive compounds	References								
	Zhao et al. (2017)	Ranneh et al. (2018)	Zulkhairi Amin et al. (2018)	Biluca et al. (2020)	Chettri & Kumari (2020)	Al-Hatamleh et al. (2020)	Silva et al. (2021)	Pimentel et al. (2022)	Rozman et al. (2022)
Trans-cinnamic acid									✓
Epicatechin									✓
Rutin									✓
Rutin hydrate									✓
Catechin		✓	✓			✓	✓		✓
Epicatechin						✓	✓	✓	
Catechol						✓		✓	
Naringin							✓		
Naringenin				✓	✓	✓	✓	✓	✓
Aromadendrin				✓	✓	✓	✓	✓	✓
Taxifolin				✓	✓	✓			✓
Isoquercetin				✓					✓
Vanillin	✓							✓	✓
Quercetin				✓	✓	✓	✓	✓	✓
Quercetine 3-O-rutinosid		✓	✓						
Isoquercetin eriodictyol					✓	✓	✓		
Syringic acid	✓	✓				✓		✓	
Syringaldehyde				✓				✓	✓

Table2.2: Continued

Bioactive compounds	References								
	Zhao et al. (2017)	Ranneh et al. (2018)	Zulkhairi Amin et al. (2018)	Biluca et al. (2020)	Chettri & Kumari (2020)	Al-Hatamleh et al. (2020)	Silva et al. (2021)	Pimentel et al. (2022)	Rozman et al. (2022)
Carnosol				✓				✓	✓
Carnosol vanillin					✓				
Scopoletin				✓			✓		✓
Eriodictyol				✓		✓	✓	✓	✓
Umbelliferon	✓			✓				✓	✓
Umbelliferone					✓				
syringaldehyde									
Hesperitin									✓
C-pentosyl-c-hexosyl-apigenin isomer									✓
Quercetin									✓
deoxyhexosyl hexoside									
Acacetin							✓		
Apigenin		✓	✓		✓	✓	✓	✓	✓
Apigenin trihexoside									✓
Kaempferol									✓
deoxyhexosyl hexoside									

Table 2.2: Continued

Bioactive compounds	References								
	Zhao et al. (2017)	Ranneh et al. (2018)	Zulkhairi Amin et al. (2018)	Biluca et al. (2020)	Chettri & Kumari (2020)	Al-Hatamleh et al. (2020)	Silva et al. (2021)	Pimentel et al. (2022)	Rozman et al. (2022)
Kaempferol	✓	✓	✓			✓	✓		✓
Isorhamnetin									✓
Luteolin						✓		✓	✓
Bis-methylated quercetin									✓
Methyl lutedin									✓
Methyl quercetin									✓
Hispidulin					✓	✓	✓	✓	✓
Chrysin		✓	✓		✓	✓	✓	✓	✓
Mirecetrin					✓	✓		✓	✓
Sinapaldehyde									✓
Sinapic protocatechuic					✓				
Pyrogallol	✓								
Vitexin-O-gallate	✓								
Isomer of vitexin-O-gallate	✓								
Acetylleugenol	✓								
Pinobanksin	✓								
Prenyl caffeate	✓								

Table 2.2: Continued

Bioactive compounds	References								
	Zhao et al. (2017)	Ranneh et al. (2018)	Zulkhairi Amin et al. (2018)	Biluca et al. (2020)	Chettri & Kumari (2020)	Al-Hatamleh et al. (2020)	Silva et al. (2021)	Pimentel et al. (2022)	Rozman et al. (2022)
24(E)-cycloart-24-ene-26-ol-3-one	✓								
Torachryson-O-hexose	✓								
Torachryson-O-(acetyl)-hexose	✓								
Torachryson-O-(galloyl)-hexose	✓								
Lapachol	✓								
20-hydroxy-24-dammaren-3-one	✓								
Mangostin	✓								
Ganoderol A	✓								
Saringosterol	✓								
Stigmasterol	✓								
Taraxerone	✓								
Abscissic acid						✓		✓	
Coniferic acid						✓		✓	
Galangin							✓		
Cinnamaldehyde									✓

2.2.1 Therapeutic Properties of Stingless Bee Honey

SBH has gained scientific interest due to its therapeutic potential, particularly in wound healing, antimicrobial activity, and antioxidant properties. While SBH research is relatively recent, honey in general has been extensively studied for its medicinal benefits. The existing literature provides insights into SBH's bioactive compounds, its mechanisms of action in oxidative stress modulation, its antimicrobial properties, and its role in wound healing. However, differences exist in the scope, methodology, and findings of these studies, highlighting the need for further investigation and clinical validation.

Several studies have focused on the bioactive composition of SBH, emphasising its phenolic and flavonoid content. Fletcher et al. (2020) reported that while many studies have explored the physicochemical and nutritional properties of SBH, only a limited number of bioactive components have been identified. Biluca et al. (2020) and Ranneh et al. (2018) identified various phenolic acids and flavonoids, including salicylic acid, *p*-coumaric acid, naringin, taxifolin, gallic acid, caffeic acid, chrysin, and 2-hydroxycinnamic acid, which contribute to SBH's antioxidant and anti-inflammatory effects. The findings also revealed significant regional variations in phenolic composition, underlining the unique nature of SBH from different geographical sources. Additionally, research from the Malaysian Agricultural Research and Development Institute (MARDI) identified protocatechuic acid (PCA) as a predominant phenolic acid, known for its role in enhancing cell proliferation during wound healing (Sujanto et al., 2021).

The antioxidant properties of SBH have been widely examined in relation to oxidative stress and cellular protection. Nur Eszaty et al. (2022) demonstrated that SBH's phenolic and flavonoid compounds effectively scavenge free radicals, stabilising harmful reactive oxygen species (ROS) and reducing oxidative damage. Al-Hatamleh et al. (2020) further linked these antioxidant effects to the modulation of signal transduction pathways, which influence inflammation, cellular survival, proliferation, and apoptosis. Chettri & Kumari (2020) explored the impact of oxidative stress on wound healing, reporting that SBH's enzymatic antioxidants, such as superoxide dismutase, and non-enzymatic antioxidants, including ascorbic acid, tocopherol, and phenolic compounds, play a crucial role in counteracting ROS. This antioxidant mechanism protects cellular components, enhances collagen production, and accelerates tissue regeneration.

The antimicrobial potential of SBH has also been a significant focus of research, particularly in its effectiveness against antibiotic-resistant bacteria. Zulkhairi Amin et al. (2018) investigated SBH's ability to prevent the growth of rifampicin-resistant *Staphylococcus aureus* (*S. aureus*), demonstrating its potential as an adjunct therapy in wound management. Rosli et al. (2020) highlighted SBH's broad-spectrum antimicrobial properties, which contribute to wound cleansing, inflammation reduction, and scar minimisation. In a comparative study, Ng et al. (2020) evaluated 46 different blossom and honeydew honeys from *Apis cerana* (honey bees) and stingless bees (*H. itama* and *G. thoracica*). Their findings indicated that all SBH samples exhibited antibacterial activity, whereas only four out of 23 honeybee honeys achieved

similar efficacy. The study further revealed that *Escherichia coli* (*E. coli*) was more susceptible to SBH's antimicrobial effects than *S. aureus*, with *H. itama* honeydew honey displaying the strongest antibacterial activity. Additionally, combining SBH with antibiotics enhanced its antibacterial efficacy, suggesting potential applications in addressing antibiotic resistance.

SBH's wound-healing properties have been investigated in relation to its anti-inflammatory effects and tissue regeneration capabilities. Jalil et al. (2017) emphasised SBH's ability to modulate inflammation, particularly in reducing scar formation during burn wound healing. Biluca et al. (2020) further explored its anti-inflammatory potential by demonstrating that SBH was non-cytotoxic at tested concentrations (1–100 μ M) and reduced nitric oxide levels and pro-inflammatory cytokine secretion in inflamed macrophages. Their study also showed that specific SBH samples enhanced interleukin-10 secretion, indicating anti-inflammatory activity. These findings suggest that SBH contains bioactive compounds that could be beneficial for managing chronic wounds and inflammatory conditions.

Another important aspect of SBH's therapeutic properties is its ability to maintain wound hydration. Nur Eszaty et al. (2022) examined SBH's humectant properties, attributing them to the presence of hydroxyl groups, sugars, proteins, lactic acid, and essential vitamins and minerals. Their study revealed that SBH facilitates an osmotic effect, which helps maintain a moist wound environment crucial for optimal healing. This finding adds another

dimension to SBH's potential in wound care, as moisture retention is a key factor in preventing infection and promoting tissue regeneration.

2.2.2 Application of Stingless Bee Honey for Wound Healing

As previously noted, SBH contains therapeutic agents that aid in wound healing, akin to those found in common honey bee honey. When it is utilised as an effective wound dressing material, it positively influences all phases of healing, contributing to improved healing outcomes by regulating both molecular and cellular mechanisms involved in cutaneous tissue repair, as depicted in Figure 2.4.

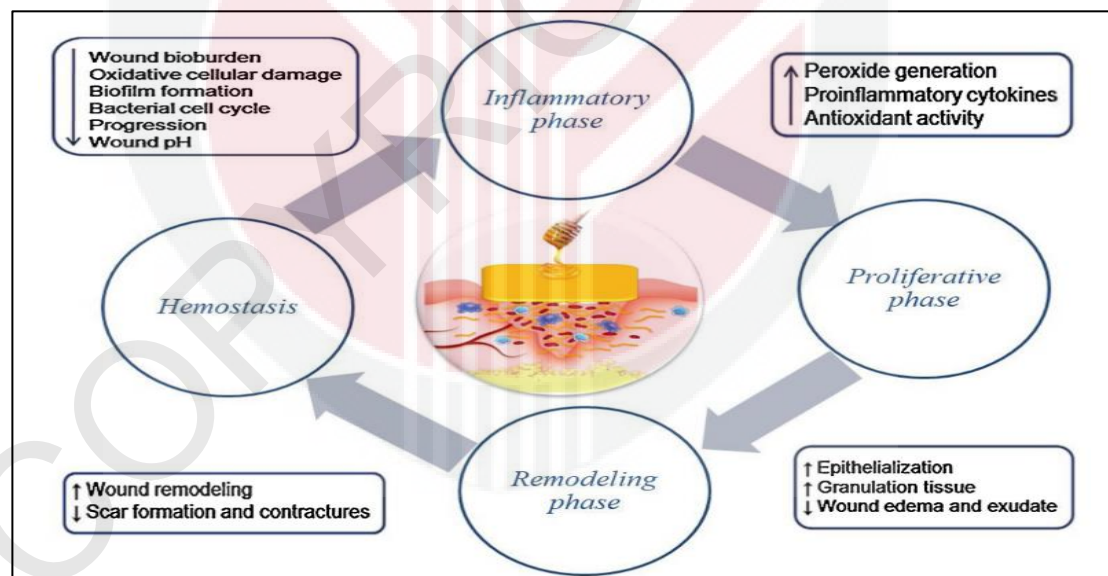


Figure 2.4: General therapeutic effect of honey in four stages of wound healing process

(Source: Krishnakumar et al., 2020).

The research on SBH presents a nuanced and multifaceted exploration of its wound healing properties across various studies. Each research team

approached the investigation from a slightly different angle, contributing to a comprehensive understanding of SBH's therapeutic potential. Marimuthu et al. (2021) laid the groundwork by identifying the key properties that make SBH effective in wound healing, focusing on its antibacterial, anti-inflammatory, and antioxidant characteristics.

Subsequent studies built upon these initial findings, each introducing unique methodological approaches and insights. Jalil et al. (2017) delved deeper into the antimicrobial and antioxidant mechanisms, while Nordin et al. (2018) took a more granular approach by examining fibroblast viability at extremely low SBH concentrations, 0.024 µg/ml. Gopal et al. (2021) innovated by exploring SBH hydrogels and their efficacy against specific bacterial strains like *Escherichia coli* (*E. coli*), demonstrating the honey's potential in advanced wound treatment formulations.

A particularly innovative contribution came from Samraj et al. (2021), who expanded the research by creating nanofibrous composite membranes combining SBH with curcumin. This approach represented a significant leap forward, showing how combining SBH with other compounds could potentially enhance its wound healing properties. Nur Eszaty et al. (2022) provided a broader perspective, examining SBH's effectiveness across various wound types, including skin ulcers, burns, and different inflammatory conditions.

The research also highlighted the complexity of SBH's composition, with Haron et al. (2022) and Sujanto et al. (2021) emphasising the impact of botanical and

geographical variations on the honey's biochemical properties. This line of research underscores the importance of standardisation to ensure consistent therapeutic outcomes. Despite these variations, a consistent thread emerged across all studies, where SBH's remarkable ability to promote wound healing through multiple mechanisms, including antibacterial action, antioxidant effects, and anti-inflammatory properties.

The progression of research reveals an increasingly sophisticated understanding of SBH's wound healing potential. From initial property identification to advanced delivery methods like hydrogels and nanofibrous membranes, scientists have systematically explored the honey's therapeutic capabilities. The studies collectively demonstrate SBH's promise in wound treatment, highlighting its ability to combat bacterial contamination, reduce inflammation, promote tissue regeneration, and support the overall healing process.

The collective research on Stingless Bee Honey (SBH) demonstrates a progressive understanding of its wound healing potential, with each study contributing unique insights that gradually unveil its complex therapeutic mechanisms. While the studies varied in methodology and specific focus, they consistently highlighted SBH's remarkable properties in wound treatment. The diverse research approaches have illuminated multiple dimensions of SBH's healing capabilities, ranging from molecular-level interactions to broader clinical implications. These investigations have not only validated the honey's

therapeutic potential but also exposed critical areas requiring further scientific exploration.

Future research trajectories appear particularly promising, with significant opportunities to bridge current knowledge gaps. Clinical validation of SBH-based wound dressings emerges as a critical next step, especially in addressing emerging challenges like antimicrobial resistance. Researchers are encouraged to develop standardized protocols that can transform these promising laboratory findings into practical medical interventions. The most compelling research directions include comprehensive in vivo studies that can definitively establish SBH's clinical efficacy. Exploring potential synergistic interactions between SBH and conventional medical treatments could unlock innovative approaches to wound care and infection management.

2.3 Nanoparticles for Wound Healing

NPs have revolutionised wound healing by offering innovative solutions that enhance traditional treatments, particularly in preventing infections and promoting tissue regeneration. Their unique physicochemical properties, including small size, large surface area, and ability to penetrate biological barriers, make them highly effective in wound dressings (Data & Kumar, 2020; Naskar & Kim, 2020). The integration of NPs into biomaterials has led to improved therapeutic outcomes by ensuring targeted drug delivery, minimising bacterial contamination, and accelerating tissue repair (Stoica et al., 2020b).

Kalashnikova et al. (2015) categorised various nanomaterials based on their impact on different phases of wound healing. Their study demonstrated that most nanomaterials play a significant role in the proliferation (Phase III) and inflammation (Phase II) stages, which are crucial for tissue regeneration. In contrast, fewer nanomaterials were found to influence haemostasis and remodelling phases, indicating that NPs can be specifically tailored for targeted therapeutic applications. This classification highlights the importance of selecting appropriate nanomaterials based on the specific needs of each wound healing stage (Figure 2.5).

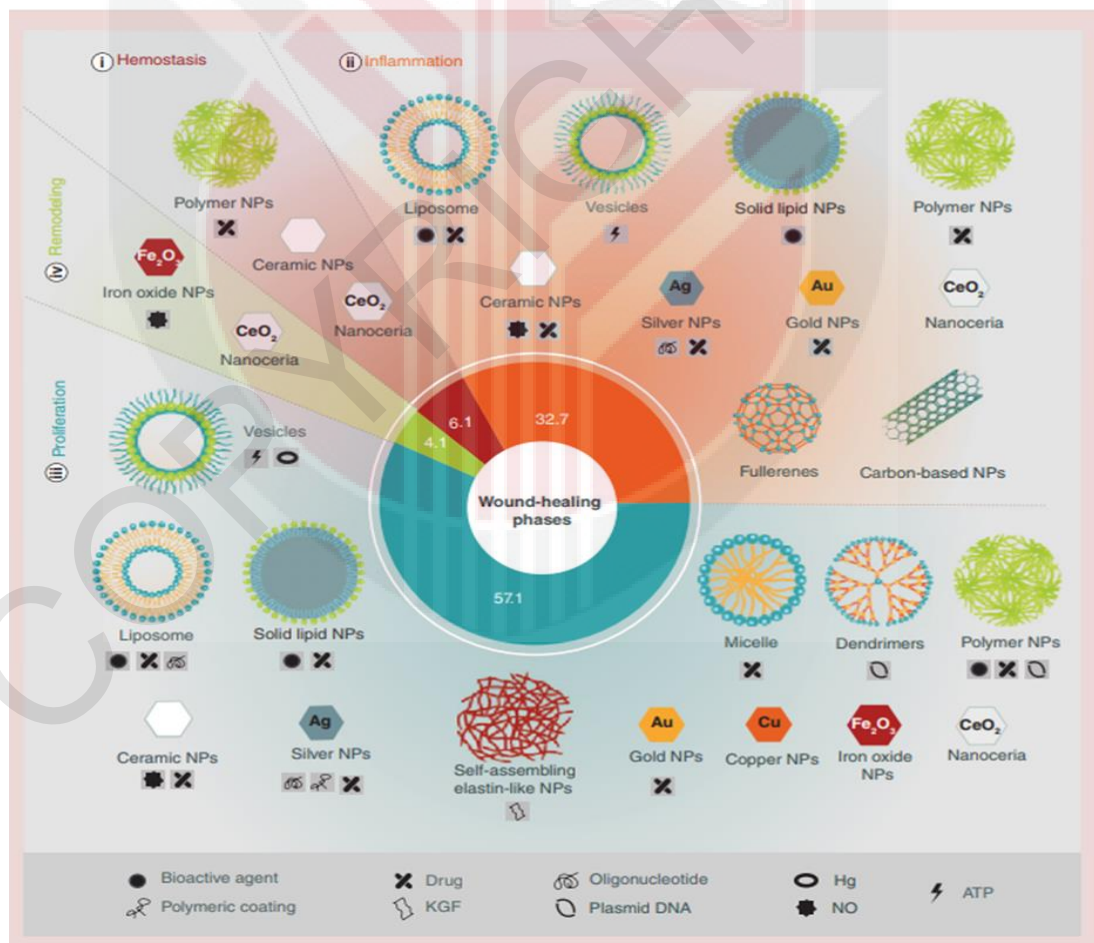


Figure 2.5: Different nanomaterials and their beneficial effects on the phase/s of wound healing
(Source: Kalashnikova et al., 2015).

Homaeigohar & Boccaccini (2020) provided further insights into the antibacterial mechanisms of NPs in wound management. Their study proposed several modes of antibacterial action, including reactive oxygen species (ROS) generation, bacterial membrane disruption, and enzyme inhibition (Figure 2.6). These mechanisms make NPs particularly effective in preventing bacterial infections and biofilm formation, which are critical challenges in chronic wound management. Metallic NPs such as Ag-NPs and gold (Au-NPs), along with metal oxide NPs like zinc oxide (ZnO-NPs) and iron oxide (Fe_2O_3 -NPs), have demonstrated strong antibacterial properties, supporting their use in wound care applications.

Azharuddin et al. (2019) and Barroso & Reis (2020) specifically focused on the antimicrobial properties of Ag-NPs, emphasising their broad-spectrum activity against antibiotic-resistant bacteria. Their inclusion in wound dressings helps maintain a sterile wound environment and reduces the likelihood of chronic infections. The study highlighted Ag-NPs' ability to prevent biofilm formation, which is a significant factor in delayed wound healing. Additionally, Bernal-Chávez et al. (2019), Chakrabarti et al. (2018), Habibullah et al. (2021), and Vijayakumar et al. (2019) explored the role of metal and metal oxide NPs in accelerating the healing process by modulating cellular responses and reducing inflammation. Their findings support the idea that incorporating NPs into wound dressings can improve patient outcomes by reducing healing time, minimising antibiotic usage, and lowering the risk of complications.

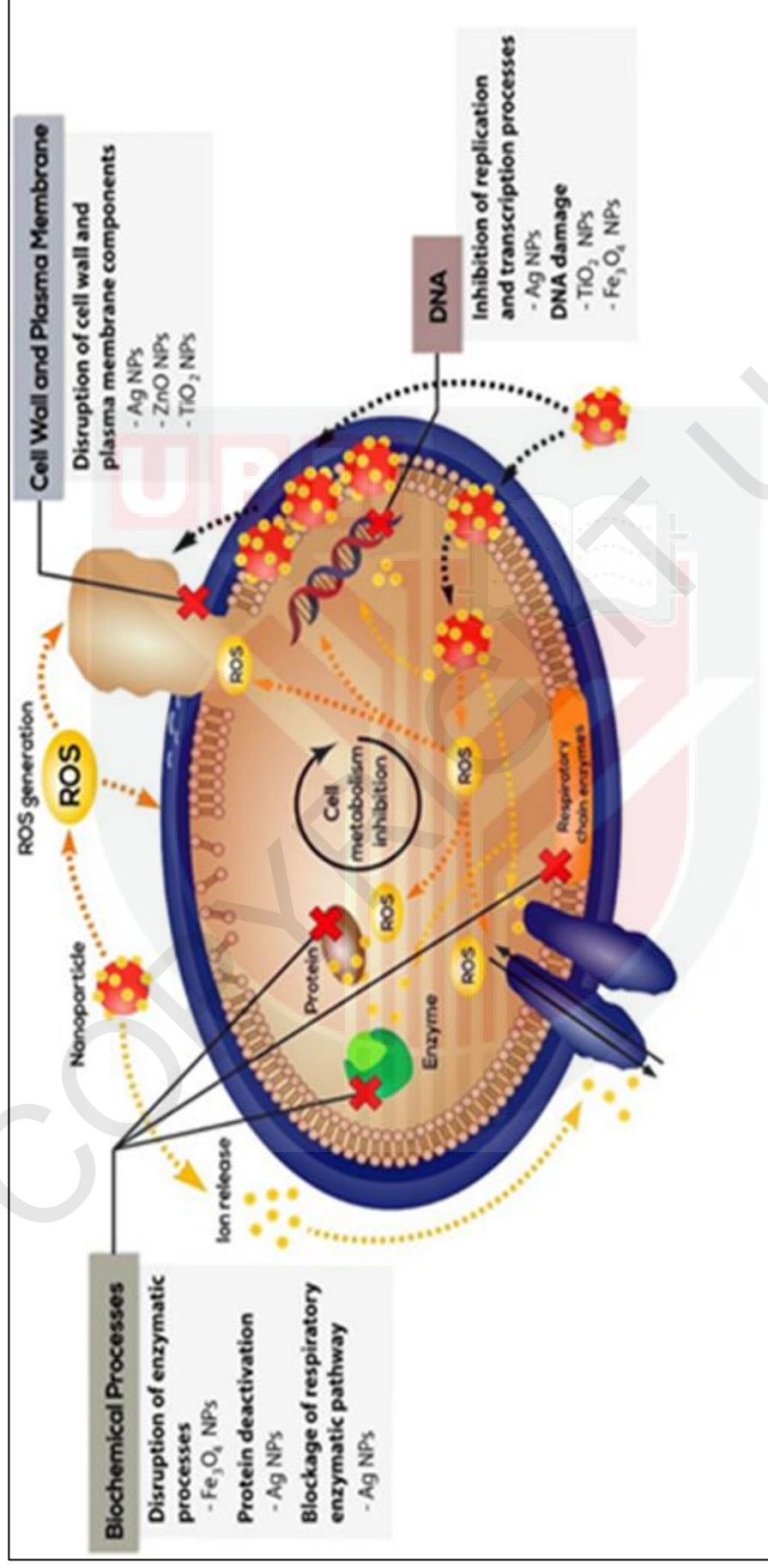


Figure 2.6: Various mechanisms suggested for the killing function of antibacterial NPs
(Source: Homaeigohar & Boccaccini, 2020).

The collective findings of these studies emphasise the multifaceted nature of NPs in wound healing, from infection control to tissue regeneration. Table 2.3 further consolidates this information by summarizing the commonly studied metal and metal oxide NPs and their mechanisms of action across different wound healing stages. The integration of these NPs into wound dressings not only enhances healing but also mitigates complications and reduces reliance on antibiotics, demonstrating their potential for improving patient outcomes



Table 2.3: Mechanisms of action of NPs for wound healing

Nanoparticles	Material	Wound Healing Phase	Mechanisms of Action	References
Metal/metal oxide NPs	Ag-NPs	Inflammation & proliferation.	- Forms a sulphuric and phosphoric bond with the bacterial cell membrane.	Kalashnikova et al. (2015); Mihai et al. (2019); Naskar & Kim (2020)
			- Once Ag-NPs have entered bacterial cells, they will undergo a transformation into low molecular weight.	
			- Ag-NPs can inhibit deoxyribonucleic acid (DNA) synthesis during cell division, enhancing bactericidal efficacy.	
Au-NPs	Inflammation & proliferation.		- Intracellular accumulation of Au-NPs inhibits ATP synthase activity, resulting in ATP depletion and subsequent bacterial cell death through impaired energy metabolism.	Data & Kumar (2020); Kalashnikova et al. (2015); Naskar & Kim (2020)
			- Au-NPs are also effective against a variety of drug-resistant pathogens via Reactive Oxygen Species (ROS) independent mechanisms.	
Copper NPs (Cu-NPs)	Proliferation		- Disruption of DNA structure.	Chen et al. (2021); Nethi et al. (2019)
			- Cellular internalisation.	
			- Accumulation in cell wall and decrease its permeability.	
			- Induction of intercellular substance leakage.	
ZnO-NPs	Haemostasis		- Keratinocyte migration is aided by cofactors in enzymatic complexes.	Abebe et al. (2020); Król et al. (2017); Vijayakumar et al. (2019)
			- Participate in the development of which perforates bacterial cell membranes, resulting in oxidant injury.	

Table 2.3: Continued

Nanoparticles	Material	Wound Healing Phase	- Mechanisms of Action	References
Metal/metal oxide NPs	Fe ₂ O ₃ -NPs	Proliferation & remodelling.	- Electrostatic and intermolecular forces mediate NPs adhesion to the bacterial cell wall, resulting in membrane depolarization and compromised structural integrity. Subsequent NPs diffusion across the membrane disrupts lipid and protein components, leading to osmotic imbalance, intracellular leakage and cellular shrinkage. - Iron ion release induces excessive ROS generation, leading to DNA replication impairment, double-strand DNA breaks, and lipid peroxidation.	Spiorescu et al. (2021)
	Titanium Oxide NPs (TiO ₂ -NPs)	Proliferation	- UV exposure exacerbates NP toxicity through ROS generation, inducing DNA damage, protein dysfunction, and metabolic impairment. NPs adhere to the cell membrane, causing structural alterations, facilitating cellular uptake, and ultimately leading to cell lysis. - NP accumulation results in localized cellular damage and amplified oxidative stress.	Bui et al. (2017); Spiorescu et al. (2021)
	Magnesium Oxide NPs (MgO-NPs)	Proliferation	- Magnesium-based NPs exhibit cytotoxicity via pH-dependent ROS generation, causing oxidative damage to cellular components. - NP surface characteristics can further inhibit bacterial cell-to-cell communication, compromising bacterial virulence.	Coger et al. (2019); Peng et al. (2021)

2.3.1 Silver Nanoparticles

Ag-NPs have garnered considerable attention in both research and industry due to their versatile and potent properties, particularly their antimicrobial capabilities. These NPs are integral to various sectors, including orthopaedics, packaging, medical devices, footwear, and household products, where their ability to inhibit microbial growth is highly valued (Bonsignore et al., 2021; Goudarzi et al., 2016). Ag-NPs are distinguished by their unique physicochemical properties, such as a high surface area-to-volume ratio and the capacity to release silver ions, which enhance their effectiveness across diverse applications.

The synthesis of Ag-NPs can be achieved through several methods, each with distinct advantages and limitations. Traditional methods, such as chemical reduction and sequential injection analysis (SIA), are widely used to produce Ag-NPs of varying shapes and sizes tailored to specific applications (El-Naggar et al., 2020). Chemical reduction is a prevalent technique where silver salts, such as silver nitrate (AgNO_3), are converted to metallic silver using reducing agents. This process involves dissolving AgNO_3 in a solvent and adding a reducing agent like sodium borohydride (NaBH_4) or citrate. NaBH_4 is an effective reducing agent but poses toxicity concerns, requiring careful handling and removal from the final product (Ye et al., 2019).

On the other hand, SIA represents an advanced method that automates Ag-NP synthesis. In this technique, AgNO_3 , a stabiliser, and a reducing agent are

sequentially introduced into a reaction coil or chamber, followed by controlled UV irradiation. This method allows for precise control of reaction parameters, improving reproducibility and efficiency. The reaction is continuously monitored using UV-Vis spectroscopy, measuring absorbance at specific wavelengths such as 435 nm to determine NPs concentration and properties (Passos et al., 2015). After synthesis, the NPs are collected and characterised using techniques like transmission electron microscopy (TEM) or scanning electron microscopy (SEM) to evaluate their size, shape, and distribution. SIA offers advantages such as automation and scalability, which enhance efficiency and reduce manual handling (Habibullah et al., 2021).

In contrast to these traditional methods, irradiation-assisted synthesis has emerged as a highly efficient alternative for producing Ag-NPs. This approach uses ionizing radiation, such as gamma rays, X-rays, or ultraviolet (UV) light, to reduce silver ions into metallic NPs. The primary benefit of this method is its ability to produce Ag-NPs with high purity and minimal by-products, as ionizing radiation directly reduces silver ions without additional chemical reducing agents (Habibullah et al., 2021). This method also provides precise control over particle size and distribution, making it especially suitable for applications requiring high-purity materials, such as in pharmaceuticals or advanced electronics.

Biosynthesis offers a green and sustainable approach to Ag-NP production by utilising biological materials as reducing agents. These materials, which include plants, bacteria, fungi, and algae, facilitate the reduction of silver ions

through their natural metabolic processes. This method not only reduces the environmental impact associated with traditional chemical synthesis but also enhances the biocompatibility of the NPs, making them suitable for various biomedical applications (Veeraraghavan et al., 2021). For instance, plant extracts contain phytochemicals that act as natural reducing agents, leading to environmentally friendly processes with reduced toxic by-products. Similarly, microorganisms can produce nanoparticles with specific properties beneficial for targeted medical applications.

2.3.1.1 Properties of Silver Nanoparticles

The morphology and dimensions of Ag-NPs are significantly influenced by factors such as reducing agents, capping agents, and reaction parameters. Precise control over these variables is essential for the controlled synthesis of Ag-NPs with desired geometric properties (Tarannum et al., 2019). Reducing agents, which facilitate the conversion of silver ions into NPs, play a crucial role in determining the size and shape of the particles. Similarly, capping agents stabilise the NPs by preventing aggregation and controlling their growth. The incorporation of growth modifiers, such as polyacrylic acid (PAA), and manipulation of pH levels further impact the nucleation and growth phases of NPs formation. Generally, variations in pH lead to morphological and dimensional changes in Ag-NPs, with larger particles typically forming under acidic conditions compared to alkaline environments (Dawadi et al., 2021).

According to Marinescu et al. (2020), the synthesis of Ag-NPs often proceeds through a two-stage reduction process. In the initial stage, a strong reducing agent rapidly generates nascent silver particles. These particles then undergo further growth through reduction by a weaker reducing agent. Nucleation begins when the solution becomes supersaturated, characterised by slower reduction and nucleation kinetics. During this phase, growth occurs through the agglomeration and diffusion of metal atoms. Surfactants play a crucial role in stabilising the NPs by preventing aggregation, reducing precursor reactivity, and slowing the reduction process. The stabilising agents form metal-surfactant complexes in the reaction solution, facilitating nucleation. Adsorbed stabiliser ions create electrostatic repulsion between coated NPs, enhancing their stability and ensuring a narrow size distribution.

In addition to reducing agents and stabilisers, synthetic parameters such as stirring duration and temperature are also critical in NPs synthesis. Typically, temperatures ranging from 20 to 100°C are used for synthesising Ag-NPs from biopolymers and plant extracts. However, temperatures exceeding 40°C can be detrimental to microorganisms involved in biosynthesis. The rate of Ag-NP synthesis increases with rising temperatures (30–90°C), which generally promotes the production of smaller NPs (Tarannum et al., 2019). The control of these parameters allows for the production of NPs with specific properties tailored to their intended applications.

The physicochemical properties of Ag-NPs, including their size, shape, and surface characteristics, directly influence their therapeutic efficacy. Smaller

Ag-NPs are known to exhibit enhanced antibacterial activity due to their increased surface area and greater ability to penetrate bacterial cells (Zahoor et al., 2021). These NPs demonstrate potent antimicrobial properties through various mechanisms. They interact directly with bacterial cells by binding to sulfhydryl groups in membrane proteins, causing disruption of the cell membrane and leading to cell death (Xiaocheng et al., 2018).

Furthermore, the small size of Ag-NPs allows them to penetrate bacterial cell walls and interfere with intracellular processes. They interact with proteins and DNA within the cells, inhibiting essential cellular functions (Naskar & Kim, 2020). Additionally, Ag-NPs release silver ions, which possess strong antimicrobial properties. These ions interact with various cellular components, contributing to bacterial cell death (Mordorski & Prow, 2016; Nešporová et al., 2020; Rajendran et al., 2018).

2.3.1.2 Application of Silver Nanoparticles in Wound Healing

The research on Ag-NPs in wound healing varies in focus, reflecting their multifaceted role in antimicrobial activity, anti-inflammatory effects, and tissue regeneration. Krishnan et al. (2020) emphasised the broad-spectrum antibacterial properties of Ag-NPs, highlighting their ability to disrupt bacterial membranes and inhibit essential cellular functions, which are crucial for preventing wound infections. This antibacterial mechanism was further explored by Kalashnikova et al. (2015), who linked the efficacy of Ag-NPs to their physicochemical properties, particularly size and shape, demonstrating

their role in enhancing tissue repair and collagen alignment. Mihai et al. (2019) extended this research by investigating the synergistic effects of Ag-NPs with tetracycline, showing a significant acceleration in wound closure through combined antibacterial action.

Beyond their antimicrobial functions, several studies have examined the anti-inflammatory properties of Ag-NPs in wound healing. Shalaby et al. (2022) focused on the bactericidal interaction between silver ions and bacterial components, showing that Ag-NP dressings reduced bacterial presence and shortened healing time without adverse effects. Bayrami et al. (2020) provided additional insight into the immune response, demonstrating that Ag-NPs evade immune system neutralisation and sustain anti-inflammatory activity. Sabarees et al. (2022) supported these findings by illustrating how Ag-NPs modulate inflammatory mediators, such as cytokines and mast cells, thereby promoting a favorable healing environment with reduced scarring.

Historical perspectives on silver's medicinal use were discussed by Bonsignore et al. (2021), who traced silver's application in wound care back to ancient civilisations, linking its traditional use to modern Ag-NP-based dressings. The study further detailed how Ag-NPs influence cellular responses, including the differentiation of fibroblasts into myofibroblasts, which enhances wound contractility and accelerates healing.

Despite these promising findings, challenges remain in the clinical application of Ag-NPs. Haidari et al. (2021) addressed concerns regarding cytotoxicity,

uncontrolled silver ion release, and aggregation within the wound microenvironment, which can impact their therapeutic effectiveness. To overcome these limitations, the study discussed the development of innovative silver-based formulations designed to improve Ag-NP stability and enhance their antibacterial efficacy.

Table 2.4 provides a summary of the various applications of Ag-NPs in wound healing, highlighting the effects of Ag-NPs size on healing period across different wound types. The diversity of these studies highlights the complexity of Ag-NPs in wound healing, underscoring their antibacterial and anti-inflammatory mechanisms while acknowledging the need for improved formulations to optimise their clinical application. Continued research is essential to refine Ag-NP-based therapies, ensuring their safety, efficacy, and consistency in wound management.

Table 2.4: Summary Application of Ag-NPs for Wound Healing

Size of Ag-NPs (nm)	Types of wound	Healing period	References
2 – 14	Second degree burn wound	28 days	Wasef et al. (2020)
25 - 50	Third degree burn wound	25 days	Jiji et al. (2020)
7 – 34	Full thickness excision wound	14 days	Diniz et al. (2020)
NA	Full thickness excision wound	14 days	Shao et al. (2019)
138	Full thickness excision wound	21 days	Fatima et al. (2021)

Table 2.4: Continued

Size of Ag-NPs (nm)	Types of wound	Healing period	References
13.9	Full thickness excision wound	7 days	Liu et al. (2017)
18.24	Full thickness excision wound	16 days	Bhubhanil et al. (2021)
20 – 50	Full thickness excision wound	11 days	Lakkim et al. (2020)
21	Second degree burn wound	14 days	Higuera et al. (2021)
10 – 30	Burn wound	18 days	Pannarselvam et al. (2017)
119 – 124	Full thickness excision wound	14 days	Lustosa et al. (2017)
7 – 70	Full thickness excision wound	14 days	Xiang et al. (2021)
NA	Diabetic wound	14 days	Scappaticci et al. (2021)
5 – 10	Full thickness excision wound	14 days	El-Aassar et al. (2020)
21 – 40	Full thickness excision and diabetic wounds	14 days (Full thickness excision wound) and 18 days (diabetic wound)	Singla et al. (2017)
5.6	Full thickness excision wound	14 days	Amirsadeghi et al. (2021)
10 – 20	Full thickness excision wound	15 days	Arya et al. (2019)
19.77	Full thickness excision wound	15 days	Shahabadi et al. (2021)
33	Full thickness excision wound	10 days	Chinnasamy et al. (2021)

Table 2.4: Continued

Size of Ag-NPs (nm)	Types of wound	Healing period	References
30 – 40	Full thickness excision wound	3 days	Viswanathan et al. (2020)
NA	Full thickness excision wound	15 days	Ye et al. (2019)

NA non-available.

2.3.2 Zinc Oxide Nanoparticles

ZnO-NPs are recognised for their versatility and wide-ranging applications in various fields, including optics, chemical sensing, semiconductors, and piezoelectrics. Their photocatalytic properties further enhance their value in purification and disinfection processes (Jin & Jin, 2019). ZnO is also notable for its biocompatibility, as the Food and Drug Administration (FDA) acknowledges zinc oxide as a generally recognised as safe (GRAS) material, which supports its use in biomedical applications (Oprea & Mihaela Panaitescu, 2020).

The synthesis of ZnO-NPs can be achieved through various methods, broadly categorised into physical and chemical techniques. Physical methods include arc plasma, thermal evaporation, physical vapor deposition (PVD), ultrasonic irradiation, and pulsed laser ablation. These methods are particularly advantageous for large-scale industrial production due to their scalability and capacity to produce high-purity ZnO-NPs. They offer precise control over NPs morphology, crystal structure, and other physical properties, making them suitable for a wide range of industrial applications (Jin & Jin, 2019).

Arc plasma involves generating a high-temperature plasma to evaporate zinc, which then condenses into NPs. Thermal evaporation uses high temperatures to evaporate zinc and deposit it onto a substrate. PVD involves the condensation of vaporized zinc onto a surface, while ultrasonic irradiation utilizes high-frequency sound waves to induce chemical reactions that produce ZnO-NPs. Pulsed laser ablation uses intense laser pulses to ablate zinc targets in a controlled environment, creating ZnO-NPs. Each of these methods allows for precise control over the size and morphology of the NPs, which is crucial for tailoring their properties for specific applications.

Chemical synthesis methods encompass a variety of liquid-phase and vapor-phase techniques. Liquid-phase methods include sol-gel, co-precipitation, microemulsions, hydrothermal, solvothermal, and sonochemical processes. Sol-gel synthesis involves transitioning a sol (liquid) into a gel (solid) to produce ZnO-NPs, while co-precipitation involves simultaneous precipitation of zinc and other reactants to form NPs. Microemulsion methods use micellar solutions to produce uniform NPs. Hydrothermal and solvothermal processes involve high-temperature reactions in aqueous or non-aqueous solvents, respectively, to create ZnO-NPs. Sonochemical synthesis employs ultrasonic waves to facilitate chemical reactions that produce ZnO-NPs. These techniques are favored for their ability to produce NPs with controlled surface properties and high purity (Oprea & Mihaela Panaitescu, 2020).

In addition to liquid-phase methods, vapor-phase techniques such as pyrolysis and inert gas condensation are used to synthesise ZnO-NPs. Pyrolysis

involves decomposing zinc compounds at high temperatures to produce ZnO-NPs, while inert gas condensation involves evaporating zinc in an inert gas atmosphere to prevent oxidation and ensure high-purity NPs production.

Beg et al. (2020) further elaborated that hydrothermal synthesis could produce ZnO-NPs with various morphologies, including flower-like, hexagonal rod-like, or spherical forms, depending on the solvent and conditions used. This flexibility in morphology makes hydrothermal synthesis particularly valuable for tailoring ZnO-NPs for specific applications.

2.3.2.1 Properties of Zinc Oxide Nanoparticles

ZnO-NPs exhibit a fascinating array of physicochemical properties that enhance their potential for biomedical applications. These NPs come in various shapes, including one-dimensional rods, two-dimensional sheets, and complex three-dimensional structures. Some ZnO-NPs even feature hierarchical porous networks, contributing to their unique functionalities and versatility in different applications (Jin & Jin, 2019).

The synthesis conditions significantly influence the physicochemical properties of ZnO-NPs. For instance, higher calcination temperatures typically result in larger particles with reduced surface areas. This observation aligns with the findings of Baharudin et al. (2018), who reported that increased calcination temperatures lead to a decrease in the surface area of ZnO-NPs, which can impact their reactivity and application. Al-kordy et al. (2021) corroborated this

trend, noting similar effects in other metal NPs, emphasising the need for precise control over synthesis conditions to tailor the properties of ZnO-NPs effectively. Additionally, Ribut et al. (2019) demonstrated that increasing the pH during synthesis reduces particle size from 59.56 nm to 46.45 nm. Interestingly, ZnO-NPs synthesised at a higher pH of 11 exhibited the largest inhibition zone (4.4 ± 0.1 cm) against bacterial growth, indicating enhanced antibacterial activity with increased pH.

These diverse physicochemical properties of ZnO-NPs translate into promising therapeutic applications. In the biomedical field, ZnO-NPs are being explored for their potential in cancer treatment, diabetes management, and combating infections. Their ability to reduce inflammation and promote wound healing is also noteworthy (Jin & Jin, 2019). One mechanism through which ZnO-NPs exert their antimicrobial effects involves disrupting bacterial cell membranes in the presence of light and generating ROS. The effectiveness of ZnO-NPs in bacterial killing is influenced by their size and concentration, with smaller particles and higher concentrations demonstrating enhanced antibacterial activity (Oprea & Mihaela Panaitescu, 2020).

Moreover, ZnO-NPs have been evaluated for their antioxidant properties. Studies indicate that ZnO-NPs exhibit lower free radical scavenging activity compared to reference standards. However, this antioxidant activity increases with higher concentrations of ZnO-NPs. The IC₅₀ value, which represents the concentration required to eliminate 50% of free radicals, is used as a measure of antioxidant potential. Lower IC₅₀ values indicate a greater antioxidant

capacity, suggesting that ZnO-NPs have potential as antioxidant agents in various therapeutic contexts (Beg et al., 2020).

2.3.2.2 Application of Zinc Oxide Nanoparticles in Wound Healing

Research on ZnO-NPs in wound healing varies in its emphasis on antibacterial mechanisms, anti-inflammatory effects, and their role in tissue regeneration. Jin & Jin (2019) focused on the fundamental role of zinc as a micronutrient in wound healing, detailing its involvement in essential processes such as clot formation, inflammation resolution, and ECM remodeling. Oprea & Mihaela Panaitescu (2020) extended this perspective by emphasising the sustained release of zinc from ZnO-NPs, which enhances cellular penetration, modulates immune responses, and promotes effective disinfection at wound sites.

The anti-inflammatory effects of ZnO-NPs were further investigated by Beg et al. (2020), who highlighted their role in suppressing inflammatory markers and enzymes while stimulating platelet aggregation and neutrophil activity, which are crucial for early wound repair. Their study also demonstrated the ability of ZnO-NPs to regulate macrophage responses, facilitate fibroblast and keratinocyte migration, and promote angiogenesis, all of which contribute to accelerated tissue regeneration. Similarly, Vijayakumar et al. (2019) examined ZnO-NPs in nanocomposite-based wound dressings, specifically focusing on their impact on keratinocyte migration and bacterial membrane perforation, which results in oxidative damage and bacterial cell death.

The antimicrobial potential of ZnO-NPs has been a key area of research. Rayyif et al. (2021) developed ZnO nano-coated dressings with enhanced antibacterial properties, demonstrating their effectiveness against wound pathogens and biofilm-associated infections. Their study reported significant bacterial inhibition within 6 to 24 hours of exposure to ZnO-NPs at concentrations of 0.6% and 0.9%. Król et al. (2017) proposed that ZnO-NPs exert antibacterial effects through hydrogen peroxide generation and electrostatic interactions with bacterial cell membranes, while the release of Zn^{2+} ions further enhance bacterial inhibition. Wang et al. (2022) expanded on this mechanism by linking ZnO-induced ROS accumulation to bacterial and fungal elimination, showing that oxidative stress contributes to microbial cell death.

Further modifications to ZnO-NPs, such as oxygen annealing, have been explored to enhance their antimicrobial activity. Kushwaha et al. (2022) reported that oxygen annealing increases ROS generation, which improves bacterial membrane disruption and overall bactericidal efficiency. Similarly, Mandal et al. (2022) emphasised the role of ZnO-NPs in compromising bacterial cell wall integrity, reinforcing their effectiveness against both Gram-positive and Gram-negative bacteria.

Table 2.5 summarises the application of ZnO-NPs in wound healing, showcasing various studies that demonstrate the effectiveness of ZnO-NPs in promoting faster and more efficient wound closure. Overall, the diversity in these studies highlights the multifaceted role of ZnO-NPs in wound healing,

with research focusing on their biological activity, antibacterial mechanisms, and material modifications to enhance efficacy. While ZnO-NPs show significant promise for wound care applications, continued research is needed to optimise their formulations and ensure clinical safety for widespread use.

Table 2.5: Summary Application of ZnO-NPs for Wound Healing.

Size of ZnO-NPs (nm)	Types of wound	Healing period	References
NA	Full thickness excision wound	16 days	Jalil et al. (2020)
37.86	Full thickness excision wound	11 days	(Batool et al., 2021)
10 – 20	Second degree burn	10 days	Melnikova et al. (2020)
4 - 6	Second degree burn	21 days	Melnikova et al. (2021)
50 – 100	Full thickness excision wound	7 days	Gutha et al. (2017)
NA	Full thickness excision wound	21 days	Metwally et al. (2020)
61	Full thickness excision wound	28 days	Arshad et al. (2019)
27	Full thickness excision wound	14 days	Khorasani et al. (2021)
NA	Full thickness excision wound	10 days	Le et al. (2022)
50 – 150	Full thickness excision wound	12 days	Norouzi et al. (2021)

Table 2.5: Continued

Size of ZnO-NPs (nm)	Types of wound	Healing period	References
20 – 40	Full thickness excision wound	10 days	Kantipudi et al. (2018)

16 – 90	Full thickness excision wound	14 days	Rasha et al. (2021)
NA	Full thickness excision wound	14 days	Zhai et al. (2018)
NA	Diabetic wound	14 days	Larijani et al. (2024)
NA	Diabetic wound	15 days	Khan et al. (2021)
40	Full thickness excision wound	14 days	Wang et al. (2023)
<100	Full thickness excision wound	14 days	Mahajan et al. (2024)
NA	Full thickness excision wound	25 days	Abbas et al. (2023)
50 -70	Full thickness excision wound	14 days	Elhabal et al. (2024)
20 – 40	Full thickness excision wound	12 days	Gao et al. (2022)
16 – 90	Full thickness excision wound	14 days	Rasha et al. (2021)

NA non-available.

2.4 Synthesis of Nanoparticles

NP synthesis has gained significant attention due to the ability to tailor surface properties and achieve high purity, making them suitable for various applications. However, conventional synthesis methods often depend on chemical agents and harsh conditions, raising environmental and toxicity concerns (Rana et al., 2020). Such methods are frequently expensive, environmentally harmful, and energy-intensive, as noted by Singh et al. (2023) and Zahoor et al. (2021). Physical synthesis can result in solvent

contamination, uneven NP distribution, and high energy consumption, while chemical methods, although effective, can lead to sedimentation, require additional purification, and generate hazardous byproducts. Scaling up these conventional approaches for industrial use also poses significant challenges (Drummer et al., 2021).

2.4.1 Green Synthesis of Nanoparticles

In response to the limitations of the conventional synthesis methods, interest in green synthesis technologies has surged. Green synthesis offers safer, cost-effective, and more sustainable methods, utilising environmentally friendly and biocompatible materials to fabricate NPs. These approaches minimise the environmental footprint of industrial processes while enabling novel applications. Muthivhi et al. (2018) have identified three key factors to consider in the bio-based synthesis of metal NPs: (i) using a non-toxic capping agent, (ii) using an environmentally friendly reducing agent, and (iii) using an environmentally friendly solvent.

Green synthesis, also known as biological synthesis, uses bacteria, fungi, yeast, plants, and algae, providing an eco-friendly option (Dikshit et al., 2021).

These methods utilise natural functional groups such as amino, carboxyl, and hydroxyl to stabilise NPs in aqueous environments. Additionally, phytochemicals in plant and algal extracts enhance the antibacterial, antifungal, and anticancer properties of the nanoparticles. This synergy combines the biological benefits of raw materials with metallic NPs, creating

multifunctional nanostructures for biomedical applications (Cruz et al., 2020). Other advantages of biological synthesis include low energy requirements, minimal waste toxicity, and cost-effectiveness. The formation of green NPs depends on the oxidation or reduction of metal ions in the biological medium used, beginning with the nucleation of metal species and culminating in the formation of NPs as precipitates within the mixture. Although the specific mechanisms for green synthesis are not yet entirely explored, the potential for sustainable operations is promising.

Fourier Transform Infrared (FTIR) analysis has revealed that compounds such as polysaccharides, polyphenols, ascorbic acid, reducing sugars, proteins, and related amino acids play a crucial role in the reduction process, with their functional groups contributing to the reaction. In a 2018 review by Vaseghi et al. (2018), the function of bioreductants and capping/stabilising agents in the green synthesis of NPs and their applications were discussed, comparing microbial synthesis and phytosynthesis in the production of green NPs.

According to Gusrizal et al. (2016), phenolic compounds found in plant metabolites are able to reduce silver ions (Ag^+) to form Ag-NPs. Hydroxybenzoic acids exhibit antioxidant activity and are suitable for forming Ag-NPs as reducing and capping agents. The carboxylate group of hydroxybenzoic acid serves as a ligand for coordination with the surface of the Ag-NPs, while the hydroxyl phenolic group acts as a reducing agent. The Ag-NPs coordinated with hydroxybenzoic acid have been shown to have a smaller size and reduced tendency for agglomeration.

The utilisation of plant-derived capping and reducing agents for NP production has proven promising. Zahoor et al. (2021) demonstrated that secondary metabolites in plant extracts, such as vitamins, polysaccharides, amino acids, proteins, enzymes, polyphenolics, and flavonoids, play crucial roles as reducing agents for Ag⁺ and as size control agents in Ag-NP formulation. Functional groups such as the carboxyl group (COOH) in glutamic and aspartic acid residues and the hydroxyl group (OH) of tyrosine residues have been discovered as key in maintaining the stability of Ag⁺ and producing small, polydisperse Ag nanoplatelets. This supports the results of Mohamad et al. (2014), who reported that hydroxyl and carboxyl groups could act as reducing agents and stabilisers in NP synthesis, while bioactive compounds such as phenolics and alkaloids play roles in capping and stabilising the produced NPs.

Several successful examples of plant-based NP synthesis have been reported. Jyoti et al. (2016) synthesised Ag-NPs using *Urtica dioica* Linn. leaves, resulting in spherical NPs 20–30 nm in size. Similarly, phenolic compounds in plant metabolites have been shown to act as reducing and capping agents for Ag-NPs, minimising aggregation and ensuring smaller particle sizes. ZnO-NPs have also been synthesised using plant extracts. For instance, *Althaea officinalis* flower extract produced ZnO-NPs with characteristic UV–vis and FTIR peaks indicating the involvement of flavonoids and other organic compounds in reduction and stabilisation processes (Elhabal et al., 2024). El-Belely et al. (2021) demonstrated the use of *A. platensis* cell filtrate to synthesise ZnO-NPs, with FTIR analysis revealing the involvement of

polysaccharides, proteins, and amino acids in capping and stabilising the nanostructures.

Materials scientists today are intensifying their search for eco-friendly reducing and capping agents that can effectively control nanoparticle reduction and stabilisation processes. The need for greener and more scalable production methods has made the biosynthesis of NPs a viable option for industrial use. Although industrial-scale applications are not yet fully developed, ongoing research continues to focus on optimising processes for sustainable large-scale implementation of honey-based and other green synthesis methods for NP production.

Among green synthesis methods, honey from sting bee or stingless bee offers distinct advantages. Unlike microbial and plant-based approaches, which often require complex processes like cell culturing and biomolecule extraction, honey can be used directly without pretreatment. Its chemical properties, including glucose, fructose, low pH, and hydrogen peroxide content, facilitate rapid and efficient NP synthesis. Honey's reducing, and capping agents enable the production of Ag-NPs and ZnO-NPs with minimal environmental impact. Studies have demonstrated that Ag-NPs synthesised with honey achieve particle sizes ranging from 5 to 98 nm (Al-Brahim & Mohammed, 2020; Czernel et al., 2021; Ghramh et al., 2021), while ZnO-NPs produced with honey exhibit sizes between 20 and 40 nm (Hoseini et al., 2015; Jeyageetha et al., 2019).

2.4.2 Stingless Bee Honey as a Bio-reducing and Stabilising Agent in the Green Synthesis of Nanoparticles.

As mention in Section 2.2, SBH has attracted increasing attention in the food, pharmaceutical, and cosmetic industries, often marketed as a potential "superfood" that commands premium prices due to its unique qualities (Shadan et al., 2018). Despite its superior nutritional and medicinal properties compared to conventional honeybee products, SBH remains underutilised. This underutilisation persists even though research suggests that stingless bee products offer more promising sources of biologically active compounds than those from honeybees, likely due to the rich vegetation found in tropical and subtropical regions where stingless bees thrive (Al-Hatamleh et al., 2020).

The distinct composition of bioactive compounds in SBH, including flavonoids, phenolics, and organic acids, makes it an excellent green resource for NP synthesis. Employing SBH for NP synthesis offers several advantages over traditional methods. It is readily available and cost-effective due to large-scale production by beekeepers, and its documented biocompatibility makes it promise for medical applications such as drug delivery. Studies have demonstrated the efficacy of SBH in generating NPs with good stability, biocompatibility, and minimal toxicity (Maringgal et al., 2020).

SBH functions as both a reducing agent and a capping/stabilising agent in the NP synthesis process (Nivethitha & Rachel, 2019). The general mechanism involves the dissociation of metal salt solution to produce positively charged metal ions (Me^{n+}). Reaction of these ions with negatively charged hydroxyl

(OH) groups of fructose and glucose present in honey to form $[\text{Me}(\text{honey})]^{n+}$ complex. Then, the addition of sodium hydroxide (NaOH) triggers formation of $[\text{Me}(\text{OH})_n/\text{honey}]$ complex, and continuous stirring and aging leads to formation of metal or metal oxide NPs.

The reducing properties of fructose, glucose, and vitamin C in honey facilitate metal NP formation by inducing glucose oxidation in an alkaline environment, resulting in gluconic acid formation (Balasooriya et al., 2017). Philip (2010) determined that NaOH addition causes Ag^+ reduction, as the alkali promotes glucose ring opening by extracting α -protons from the oxygen of the sugar ring, while metal ions oxidize glucose to gluconic acid.

Beyond simple sugars, enzymes and proteins in honey contribute significantly to NP synthesis. Glucose oxidase catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide, potentially leading to metal oxide formation. Similarly, catalase and peroxidase can catalyze hydrogen peroxide breakdown, also contributing to metal oxide formation. Proteins act as capping agents, surrounding NPs and preventing agglomeration or oxidation (Haiza et al., 2013). These proteins influence size, shape, and stability of NPs by controlling nucleation and growth rates.

On the other hand, phenolic acids in honey contribute to NP formation through synergistic effects. They serve as effective reducing agents due to their multiple hydroxyl groups and as efficient dispersants due to their derived $\text{C}=\text{O}$ groups (Habsi et al., 2019; Liu et al., 2018). These properties enable phenolic

acids to prevent NP aggregation, enhancing stability by keeping particles well-dispersed and less prone to precipitation.

2.4.3 Stingless Bee Honey Nanoparticles

A review of the literature reveals that only a few studies have explored the use of SBH in NP synthesis. Maringgal et al. (2020) were among the pioneers in this area, successfully biosynthesising calcium oxide NPs (CaO-NPs) using *Trigona sp.* honey. The resulting spherical NPs had an average size below 100 nm, with a height of 2.3 nm. These NPs exhibited strong antifungal activity, effectively inhibiting anthracnose disease at a 15% concentration. Additionally, cytotoxicity evaluations confirmed their biocompatibility, showing no toxicity to MRC-5 and VERO cells. Their findings highlighted the potential application of nanotechnology in the fresh produce industry. The synthesis process involved depositing and precipitating calcium carbonate (CaCO_3) in *Trigona sp.* honey. Specifically, CaCO_3 powder was stirred in distilled water for 5 h at room temperature, followed by filtration. The honey was then added to facilitate molecular binding, and the mixture was aged for 3 days before being calcined at 900 °C for one hour. The physicochemical characteristics of the synthesised CaO-NPs were thoroughly analysed using field emission scanning electron microscopy (FESEM), atomic force microscopy (AFM), X-ray diffraction (XRD), and Fourier-transform infrared spectroscopy (FTIR).

Building upon this research, Rozman et al. (2021) developed SBH-based nanoemulsions (NEs) using a high-pressure homogenisation technique. The

resulting NEs exhibited particle sizes ranging between 10 and 100 nm. Through response surface methodology (RSM) optimisation, oleic acid was identified as a key factor influencing both particle size and the polydispersity index (PDI). The developed models demonstrated strong predictive accuracy, with R^2 values exceeding 0.90. Furthermore, FTIR analysis confirmed the preservation of SBH's functional groups, while transmission electron microscopy (TEM) provided detailed morphological insights into the nanoemulsions.

More recently, Al-Hatamleh et al. (2024) advanced the application of SBH in nanotechnology by formulating SBH-loaded alginate NPs (H-ALG NPs) using an ionic crosslinking method. These NPs had an average size of 312 ± 4.32 nm, with an encapsulation efficiency of 84.74% and a loading capacity of 23.12%. The study demonstrated that H-ALG NPs exhibited dose-dependent antioxidant activity, low cytotoxicity, and enhanced cell migration in human dermal fibroblasts. In vivo studies using a mouse wound model revealed that the NPs significantly accelerated wound healing, as evidenced by improved histological features. Gene expression analysis further confirmed their role in promoting proinflammatory mediators essential for wound repair. The characterisation of H-ALG NPs was conducted using TEM and SEM for morphological analysis, quantitative polymerase chain reaction (qPCR) for measuring mRNA expression of proinflammatory mediators, and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay for evaluating antioxidant activity. Cytotoxicity was assessed using the MTT assay on human dermal fibroblast cells.

A key inconsistency arises when comparing Al-Hatamleh et al. (2024) with Maringgal et al. (2020) and Rozman et al. (2021). While Maringgal et al. (2020) and Rozman et al. (2021) focused on the physicochemical characterisation of SBH-derived nanomaterials, Al-Hatamleh et al. (2024) provided a more comprehensive biological evaluation, including cytotoxicity, antioxidant activity, and wound-healing efficacy. However, together, these studies underscore the growing interest in leveraging SBH for NP synthesis and biomedical applications. From antifungal activity to wound healing potential, the findings reinforce SBH's versatility in nanotechnology, paving the way for future innovations in medicine, agriculture, and biotechnology.

2.5 Summary

Chapter 2 provides an in-depth review of the literature on wound healing, covering different types of wounds and the complex healing process, along with the crucial role of wound dressings. It explores the therapeutic properties of SBH, emphasising its antibacterial, anti-inflammatory, and antioxidant effects, as well as its application in wound dressings to enhance healing. Additionally, the chapter examines the properties and wound care applications of Ag-NPs and ZnO-NPs. Ag-NPs are highlighted for their strong antimicrobial activity and ability to accelerate wound closure, while ZnO-NPs are recognised for their antibacterial properties, immune-modulating effects, and role in tissue regeneration. The chapter concludes with a discussion on the advantages of green synthesis, focusing on the use of SBH as a bio-reducing and stabilising agent, and reviewing existing research utilising this eco-friendly approach.

Future research should focus on the synthesis of NPs and their potential as materials for wound dressings that not only overcome the limitations of conventional dressings but also serve as an effective solution for wound-related challenges.



CHAPTER 3

METHODOLOGY

3.1 Research Design

Research activities presented in this chapter were designed to achieve the objectives of the study. Figure 3.1 shows the overall structure of methodology performed in this study.



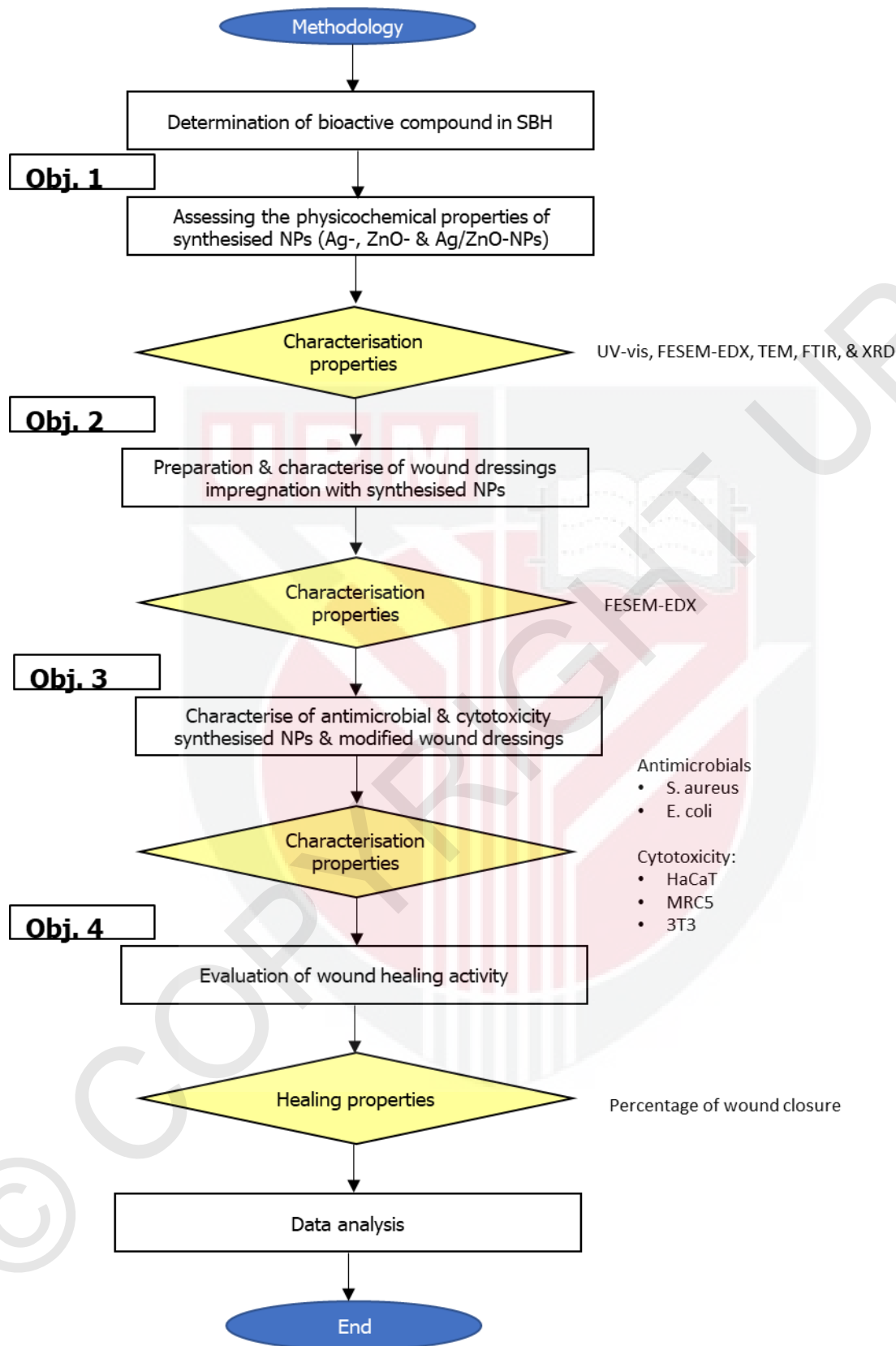


Figure 3.1: Flowchart of the research works.

3.2 Materials

The SBH utilised in this study was sourced from the species *H. itama* and was purchased from a local market situated in Serdang, Selangor, Malaysia. This particular SBH was carefully collected from a diverse array of multifloral botanical sources, ensuring a rich and varied composition of nutrients and bioactive compounds. To ensure the honey met the quality standards outlined in the Malaysian Standard for Kelulut (Stingless Bee) honey (MS 2683:2017), it was subjected to a drying process at a controlled temperature of 40 °C to effectively remove any excess water content. The chemicals employed in this study included Sodium Hydroxide (NaOH, 98%, Sigma Aldrich), Silver Nitrate (AgNO_3 , $\geq 99.0\%$, Sigma Aldrich), Zinc Nitrate ($\text{Zn}(\text{NO}_3)_2$, 98%, Sigma Aldrich), Dimethyl Sulfoxide (DMSO), Ethanol (MERCK), Exigo diluent RFID, Exigo Lyse RFID, and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), all of which were procured from a reputable local chemical supplier in Malaysia. Additionally, various cell lines including human lung fibroblasts (MRC5), human keratinocytes (HaCaT), and mouse embryonic fibroblasts (3T3) were employed in the study, alongside bacterial strains such as *Staphylococcus aureus* (ATCC 43300) and *Escherichia coli* (ATCC 25922). These biological materials were generously provided by the Institute of Bioscience (IBS) at Universiti Putra Malaysia, ensuring high-quality and reliable sources for the research. For the animal study component, a total of 30 white Sprague-Dawley rats were obtained, along with the necessary mouse pellets and bedding, from a local veterinary and medicament supplier in

Malaysia, ensuring ethical standards and proper care for the animals involved in the research.

3.3 Determination of Bioactive Compounds of Stingless Bee Honey

Determination of bioactive compounds of SBH was performed on an ultra-high-performance liquid chromatography quadrupole time of flight/mass spectrometry (UHPLC-Q-TOF/MS). The UHPLC analysis was performed on an Agilent 1290 UHPLC system (Agilent, USA) equipped with an Agilent ZORBAX SB-Aq C18 column (2.1x150 mm, 3.5 μ m) at 30 °C system. preparation and parameter used were adopted from Zhao et al. (2017). The parameters used for the analysis were listed in Table 3.1.

Table 3.1: UHPLC-Q-TOF/MS Analysis Parameters

Parameter	Value
Sample Preparation	1 mL SBH dissolved in 1 mL 90% methanol (v/v), filtered through 0.2 μ m nylon membrane
Column Temperature	30 °C
Mobile Phase A	0.1% (v/v) aqueous formic acid
Mobile Phase B	Methanol
Gradient	Linear gradient as follows: 0-5.0 min: 1% B (v/v) 5.0-30.0 min: 1-30% B (v/v) 30.0-40 min: 30-95% B (v/v) 40.0-58.0 min: 95% B (v/v) 58.0-60.0 min: 95-1% B (v/v) 60.0-80.0 min: 1% B (v/v)
Flow Rate	0.3 mL/min
Injection Volume	1 μ L
MS System	Agilent 6545 Accurate-Mass Q-TOF/MS
Ionization Source	Electrospray ionization (ESI)
Drying Gas	Nitrogen
Drying Gas Flow Rate	10.0 μ L/min
Drying Gas Temperature	350 °C

Table 3.1: Continued

Parameter	Value
Drying Temperature	Gas 350 °C
Nebuliser Pressure	40 psi
Capillary Voltage	3500 V (negative), 4000 V (positive)
Fragmentor Voltage	130 V (positive and negative)
Collision Energies	40 V (positive MS/MS), 20 V (negative MS/MS)
Mass Range	m/z 100-1500
Mass Accuracy	≤ 5 ppm

By utilising this advanced technique, we aimed to identify and quantify the various bioactive constituents within SBH, which could potentially contribute to its therapeutic properties and efficacy in various applications. The results obtained from this analysis will provide valuable insights into the chemical composition and bioactivity of SBH, thereby facilitating further research and development in this field.

3.4 Synthesis and characterisation of Silver, Zinc Oxide, and Bimetallic Silver/Zinc Oxide Nanoparticles using Stingless Bee Honey

3.4.1 Synthesis of Silver, Zinc Oxide, and Bimetallic Silver/Zinc Oxide Nanoparticles using Stingless Bee Honey

The method employed in this study was adapted from previous investigations conducted by Maringgal et al. (2020) and Bayrami et al. (2020), incorporating slight modifications to enhance the overall process. To begin, 10 g of AgNO₃ was thoroughly mixed with 10 g of SBH, and this mixture was subsequently diluted to a final volume of 100 mL using distilled water. A concentrated 5 M NaOH solution was then gradually introduced into the mixture to adjust the pH to approximately 11, all while ensuring vigorous stirring to facilitate a uniform

reaction. The reaction mixture was heated to 80°C and maintained at this temperature for a duration of 2 hours, followed by an overnight storage period at room temperature to allow for further reaction and stabilisation. The resulting precipitate was meticulously collected through centrifugation at 4000 rpm for 20 minutes, washed multiple times with distilled water and ethanol to remove any impurities, and finally dried at 60°C for a continuous period of 24 hours to ensure complete evaporation of solvents. The synthesis of ZnO-NPs adhered to the same procedural framework as that of the Ag-NPs, with the sole modification being the replacement of AgNO₃ with Zn(NO₃)₂ as the precursor material. In parallel, the preparation of bimetallic Ag/ZnO-NPs was carried out using a similar methodological approach, with a carefully predetermined weight ratio of AgNO₃ to Zn(NO₃)₂ set at 1:1 (5 g of AgNO₃ and 5 g of Zn(NO₃)₂). The selection of this weight ratio is to ensures consistency and reliability across all synthesised NP types, in order to achieve the desired nanostructure characteristics (Sumbal et al., 2019). A visual representation of the synthesis process is provided in Figure 3.2.

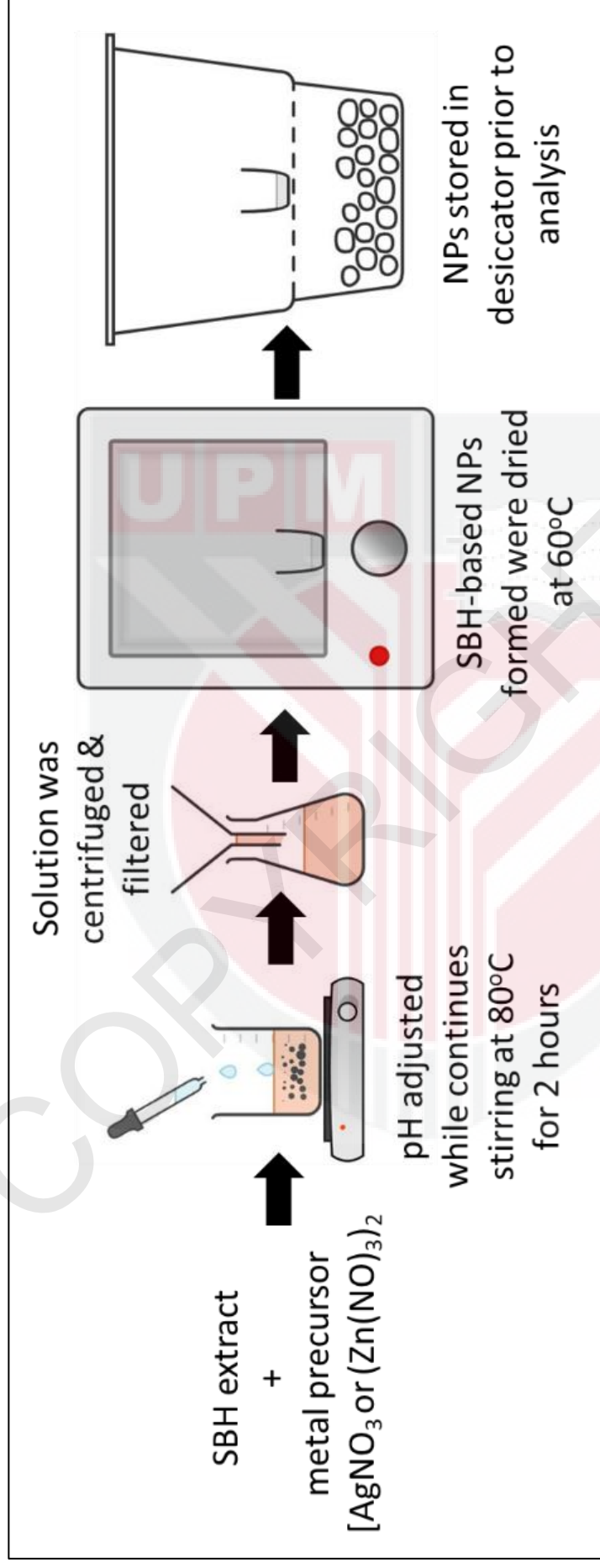


Figure 3.2: Synthesis of NPs using SBH.

3.4.2 Characterisation of Silver Nanoparticles, Zinc Oxide Nanoparticles and Bimetallic Silver/Zinc Oxide Nanoparticles

3.4.2.1 Ultraviolet–Visible Spectroscopy

The formation of the synthesised Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs was evaluated by UV-Vis spectrophotometer (Hach DR/4000, USA). The NPs solutions were scanned from 280 to 600 nm at a medium rate and distilled water was considered as the blank sample. This technique analyses the light absorption properties of the NPs, which can provide evidence of their successful synthesis.

3.4.2.2 Field Emission Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy

The size and shape of the synthesised NPs were investigated using a field-emission scanning electron microscope (FESEM; JOEL JSM-7600F, USA) operated at an accelerating voltage of 5 kV, a working distance of 3.9 mm, and magnifications of 20,000x, 50,000x, and 100,000x. To prevent electrostatic charging artifacts during imaging, the samples were coated with a thin layer of platinum prior to analysis. The elemental composition of the NPs was determined using energy-dispersive X-ray spectroscopy (EDX) coupled with the FESEM instrument. This technique allows for the identification and quantification of the elements present within the NPs.

3.4.2.3 Transmission Electron Microscopy

Transmission electron microscopy (TEM; Hitachi H-7100 STEM, USA) was employed to investigate the potential encapsulation of the synthesised NPs by bioactive compounds present in SBH. The instrument was operated at an accelerating voltage of 120 kV, achieving a high resolution of 2.5 Å. The scanning was conducted at the magnification of 25,000x, 50,000x, and 100,000x. To prepare the samples for TEM analysis, the SBH-based NPs were re-dispersed in deionised water. A small droplet of this suspension was then deposited onto a copper grid for analysis. Subsequently, the solvent was evaporated using an infrared lamp prior to capturing the TEM images. This step ensures the NPs remain on the grid for visualisation.

3.4.2.4 Fourier Transform Infrared Spectroscopy

Fourier-transform infrared (FTIR) spectroscopy (Spectrum One, Perkin Elmer, Germany) was employed to identify potential biomolecules in the SBH solution responsible for capping and stabilising the NPs. The FTIR spectra were collected in the mid-infrared region, ranging from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} .

3.4.2.5 X-Ray Diffraction Spectroscopy

XRD has been used to determine the crystalline structure of the synthesised NPs. The diffraction patterns indicate the presence of specific crystal planes,

confirming the formation of crystalline NPs. The XRD patterns were acquired using a SHIMADZU XRD6000 diffractometer over a 2θ range of 20° to 70° . Continuous scanning was performed at a scan rate of $2^\circ/\text{min}$ with a step size of 0.02° . Crystallite sizes of Ag-NPs, ZnO-NPs, and Ag/ZnO-NPs were determined from the broadening of the diffraction peaks using the Debye-Scherrer equation as described in Eq. 1:

$$\text{Particle size} = \frac{0.9 \times \lambda}{\beta \cos \theta} \quad \text{----- (1)}$$

where λ is the wavelength of X-rays that interacted with the basis (nm), β is the full width at the half maximum (radius), and θ is the angle of diffraction ($^\circ$).

3.5 Preparation and Characterisation of Wound Dressings Impregnated with Synthesised Nanoparticles

3.5.1 Preparation of Wound Dressings Impregnated with Synthesised Nanoparticles

The 2.5×2.5 cm sections of wound gauze were impregnated by immersing them in a prepared mixture comprising synthesised NPs and an SBH/ethanol solution in a 10:1 ratio. This specific mixture was chosen to ensure effective adhesion of the synthesised NPs to the cotton surface, thereby enhancing the gauze's therapeutic properties (Bayrami et al., 2020). To further enhance the uniformity of the distribution, the gauze underwent a rigorous ultrasonic treatment for 40 minutes at a controlled temperature of 30°C . This step was crucial in achieving a homogeneous distribution of the synthesised NPs throughout the cotton fabric, ensuring that the therapeutic agents would be

evenly available for potential application. Once the ultrasonic treatment was complete, the treated gauze was carefully placed on a sterile petri dish. It was then dried in an oven set at 37°C for a duration of 4 hours, a process aimed at eliminating any residual moisture that could compromise the integrity and effectiveness of the nanoparticles. After drying, the treated gauzes were stored in a sterile desiccator, ensuring a controlled environment to maintain their properties for subsequent analysis and experimentation (Figure 3.3). This preparation process is essential for ensuring the gauze's efficacy in wound care applications.

3.5.2 Characterisation of the wound dressings impregnated with Synthesised Nanoparticles

The dispersion of the NPs over the dressings was determined using FESEM (JOEL JSM-7600F, USA) operated at an accelerating voltage of 15 kV and the chemical composition of the treated dressing was recorded using an EDX detector.

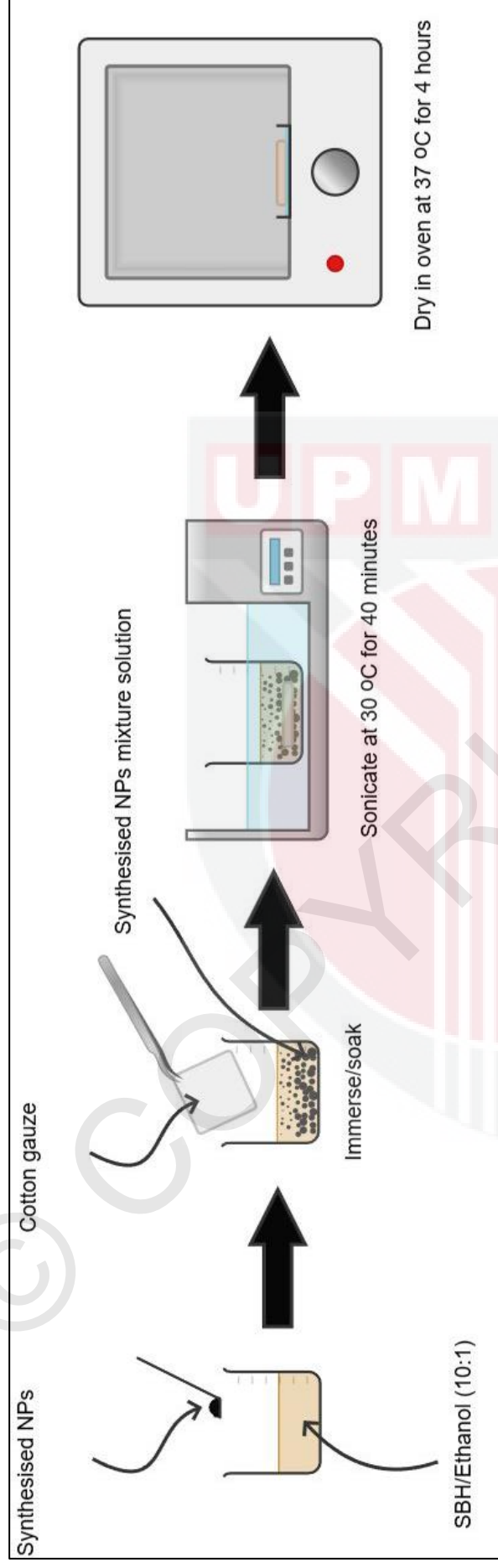


Figure 3.3: Summary of the preparation of the modified cotton gauze comprising synthesised NPs

3.6 Characterisation of Antimicrobial and Cytotoxicity of Synthesised Nanoparticles and Wound Dressings Impregnated with Synthesised Nanoparticles

3.6.1 Antibacterial Assay of Synthesised Nanoparticles and Wound Dressings Impregnated with Synthesised Nanoparticles

The antibacterial activity of the green-synthesised NPs and the modified wound dressings was tested against two notable bacterial strains: *S. aureus* (ATCC 43300) and *E. coli* (ATCC 25922). These strains were chosen due to their significant relevance in both clinical and healthcare settings, often being responsible for a variety of infections that can complicate wound healing. Chlorhexidine hydrochloride was utilised as the positive control to evaluate the antimicrobial activity of the synthesised NPs, as it is a well-established antiseptic known for its effectiveness against a broad spectrum of microorganisms. In parallel, commercial Durafiber Ag was employed as the positive control for assessing the antimicrobial performance of the wound dressings infused with the synthesised NPs. This comparative analysis not only highlights the potential of green-synthesised NPs as effective antibacterial agents but also aims to determine their efficacy in enhancing the overall performance of wound dressings. By examining these interactions, the study seeks to contribute valuable insights into innovative wound care solutions that harness the benefits of nanotechnology in combating bacterial infections.

3.6.1.1 Agar Well Diffusion method for Silver, Zinc Oxide, and Bimetallic Silver/Zinc Oxide Nanoparticles

The antimicrobial activities of the prepared NPs were assessed using the diffusimetric method, following the guidelines established by the Clinical Laboratory and Standards Institute Inc. (CLSI). The bacteria were streaked on Luria Bertani (LB) agar media and incubated at 37°C for 24 hours. To prepare the colony suspension, 10-20 isolated colonies were selected and mixed with 5 mL of saline solution (0.85 % NaCl). The turbidity of the suspension was adjusted to match the 0.5 McFarland standard. The resulting inoculum was thoroughly vortexed and spread on LB agar by streaking the swab in three consecutive passes, each at a 60° angle to ensure even distribution. Wells with approximately 5 mm diameters were created and sealed with sterile cork borers. The wells were filled with NPs samples at a concentration of 200 µg/ml, while 100% dimethyl sulfoxide (DMSO) served as the negative control. The plates were incubated overnight at 37°C, with the incubated side facing upward, following an initial 2-hour incubation at room temperature. The zone of inhibition (ZOI) was calculated by measuring the growth inhibition around the wells. Each experiment was repeated three times to ensure consistent results.

3.6.1.2 Agar Disc Diffusion Method for Wound Dressings Impregnated with Synthesised Nanoparticles

S. aureus and *E. coli* were cultured overnight in Luria-Bertani (LB) broth at 30°C with orbital shaking. Bacterial cells were harvested and resuspended in phosphate-buffered saline (PBS). The bacterial suspensions were

incorporated into molten LB agar and poured into sterile petri dishes. After solidification, sterile disks impregnated with either methanol (control) or test fractions were placed on the inoculated agar plates. Plates were incubated at 30°C for 16 hours, and the diameters of the resulting zones of inhibition (ZOI) were measured to assess antimicrobial activity.

3.6.1.3 Determination of the Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) was measured by two-fold serial dilution method (Asan et al., 2022). A bacterial suspension equivalent to a 0.5 McFarland standard was prepared and diluted 1:20 in LB broth. In a microtiter plate, hundred μL of inoculum dispense into well in column 1 until 11 and hundred μL of LB broth into well in column 12 as sterility control. 100 μL the extract was added and mixed into column 1 and serially diluted until column 10 and discarded 100 μL of the solution from column 10. Column 11 was used as growth control. The plate was incubated at 37°C for 24 hours. The MIC was defined as the lowest extract concentration that inhibited visible bacterial growth.

3.6.1.4 Determination of the Minimum Bactericidal Concentration

To determine the minimum bactericidal concentration (MBC), 10 μL aliquots from the wells exhibiting no visible growth in the MIC test were subcultured onto LB agar plates. The plates were incubated at 37°C for 24 hours. The MBC was defined as the lowest concentration of the extract that resulted in no

colony growth on the agar plate. Column 1 until 10 was indicated the concentration of the extract 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.13 µg/mL, 1.56 µg/mL, 0.78 µg/mL and 0.39 µg/mL, respectively. The solution in column 12 was used as sterility control and 11 as growth control. It was incubated for 24 h at 37°C. The MBC value was determined by the lowest concentration that showed no observable growth on agar plates.

3.6.2 *In-vitro* Cytotoxicity

The cytotoxicity of Ag-NPs, ZnO-NPs, and Ag/ZnO-NPs was evaluated using the MTT assay. A cell culture with a density of 4×10^3 cells/mL was prepared and seeded into 96-well plates at a volume of 100 µL per well. Serial dilutions of the NPs samples were prepared at concentrations of 100, 20, 10, 5, and 1 µg/mL, and added to the cell cultures. The plates were incubated for 72 hours. After incubation, MTT solution was added to each well, and the plates were further incubated for 3 hours to allow for formazan crystal formation. The formazan crystals were solubilised with dimethyl sulfoxide (DMSO), and the absorbance was measured at 570 nm using an ELISA reader. The IC₅₀ value, representing the concentration of NPs causing 50% cell growth inhibition, was determined from the dose-response curve and cell viability was determined using the equation given below:

$$\text{Cell viability} = \frac{\text{Absorbance sample (mean)}}{\text{Absorbance control (mean)}} \times 100 \% \quad \text{-----(2)}$$

3.7 Evaluation of *In-vivo* Wound Healing Activity

3.7.1 Wound Closure

Thirty male Sprague-Dawley rats, aged 8 weeks and weighing approximately 250 ± 30 g, were assigned to six distinct groups, with six rats in each group. The rats were housed under meticulously controlled environmental conditions, maintaining a temperature of 25 ± 2 °C, relative humidity at 55%, and a 12-hour light/dark cycle to replicate natural living conditions. They were provided ad libitum access to standard laboratory pellets and fresh water to ensure their health and well-being throughout the study. The experimental protocol was reviewed and approved by the Animal Ethics Committee of MARDI (authorisation number: 20230622/R/MAEC00138). Before initiating the study, the rats underwent a one-week acclimatization period to adapt to their environment. Following this period, they were anaesthetised using carbon dioxide (CO₂) to minimise stress and discomfort during the procedures. Dorsal fur was carefully shaved to create a uniform surface for the subsequent wound creation process. Two full-thickness skin wounds, each 8 mm in diameter, were then surgically created on the dorsum of each rat, as depicted in Figure 3.4.

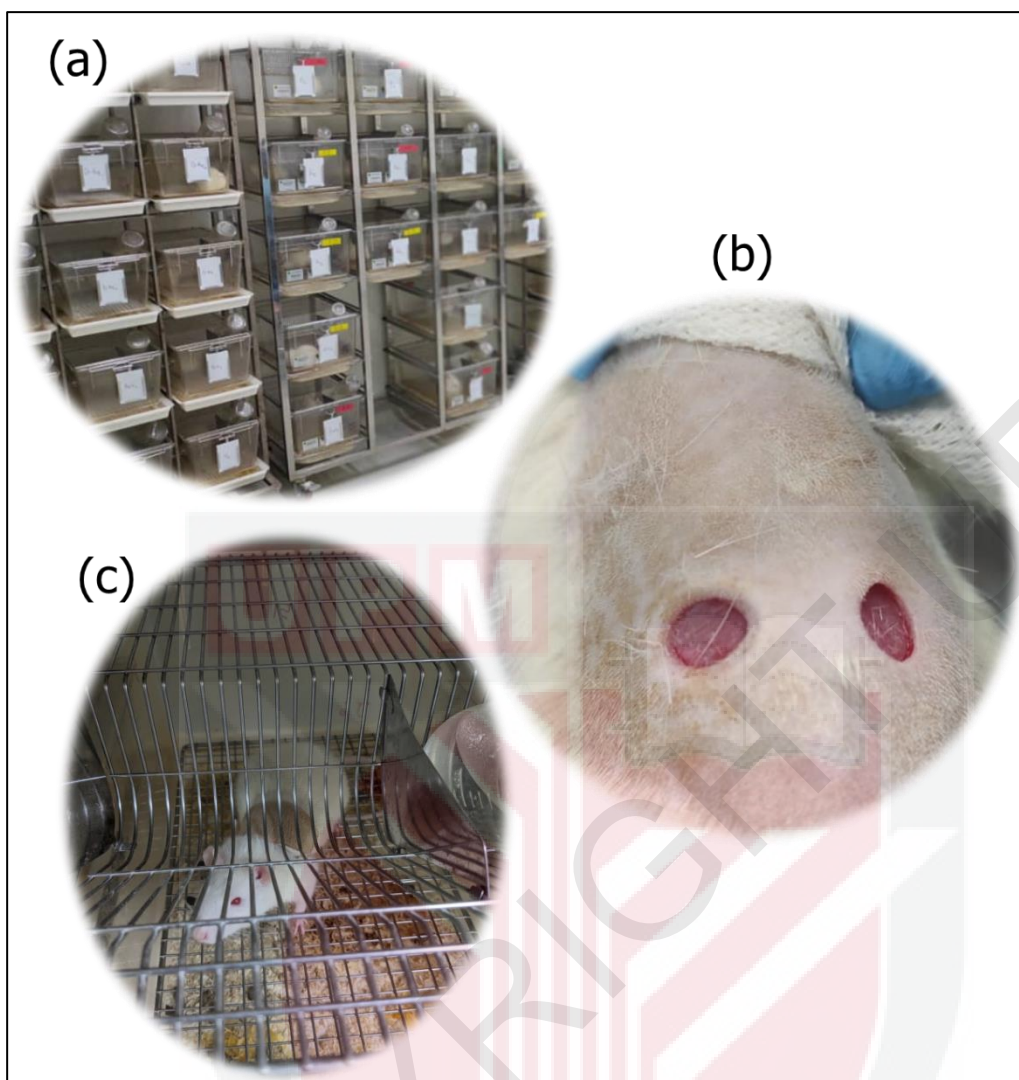


Figure 3.4: *In-vivo* wound healing activity; (a) individual cages for each rat, (b) circular excisional wounds (8 mm) on the dorsum, and (c) topical application of treated gauzes on the wound to evaluate their wound healing properties.

The objective of the study was to evaluate the wound healing efficacy of modified gauze impregnated with synthesised NPs compared to commercially available gauze and a negative control. Treatments were applied systematically according to the experimental groups outlined in Table 3.2. For the commercial comparison, Durafiber Ag was chosen due to its proven antimicrobial properties, which are effective against a broad spectrum of common wound pathogens. This antimicrobial activity is essential in reducing

bacterial bioburden and mitigating the risk of wound infection, factors critical to promoting effective wound healing. Distilled water served as the negative control to establish a baseline for healing in the absence of active therapeutic agents.

Table 3.2: List of classified rats based on the treated gauzes

Group	Treatments
1	Negative Control (distilled water)
2	Positive Control (Commercial Durafibre Ag)
3	Ag-NPs/SBH
4	ZnO-NPs/SBH
5	Ag/ZnO-NPs/SBH

Then, the animals were kept in individual cages for 20 days. The wound size was monitor and measured on the day 0, 4, 8, 12, 16, and 20 (Figure 3.5). The percentage closure will be calculated as equation 3:

$$\text{Percentage of wound closure, \%} = \frac{(A_z - A_t)}{A_z} \times 100 \quad \text{-----(3)}$$

where A_t is the wound area on corresponding days and A_z is the wound area on day 0.



Figure 3.5: Measurement of wound size.

3.7.2 Histopathological Analysis

Skin tissues from the wound area were collected on the 20th post-injury day. Tissue samples were fixed in 4% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Microscopic images of the sections were captured using a Leica DM750 (Germany), light microscope. Histopathological assessment focused on evaluating epithelialisation, vascularisation, and fibrosis within the wound area for each experimental group, ensuring a comprehensive analysis of the final tissue response and the complete healing process.

3.7.3 Blood Biochemical and Haematological Analyses

Blood biochemical and haematological determinations were measured on day 20 for all groups. Blood biochemical analysis was performed using the Dirui CS-300B Auto-Chemistry Analyser, China, meanwhile the haematological analysis was conducted using Exigo™ H400 veterinary Haematology Analyser, Sweden. The blood samples for biochemical was centrifuged at 2200 to 2500 rpm for 15 min to create a blood serum before being analysed using Auto-Chemistry Analyser.

3.8 Data Analysis

Data analysis was conducted using RStudio Software (Version 4.3.0). Prior to further analysis, normality and homogeneity of the variables were assessed. Analysis of Variance (ANOVA) was employed to compare means across multiple groups, and Tukey's Honestly Significant Difference (HSD) test was used to analyse differences between groups, and a significance level of $p < 0.05$ indicated a significant difference between groups.

3.9 Summary

This chapter outlines the comprehensive methodology employed in the study, beginning with the analysis of bioactive compounds in SBH using UHPLC-Q-TOF/MS. Following this, the synthesis of Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs was carried out using SBH as a reducing and stabilising agent.

The synthesised NPs were characterised using various advanced techniques, including UV-Visible spectroscopy, FESEM-EDX, TEM, FTIR, and XRD to determine their physicochemical properties. Antibacterial efficacy of the synthesised NPs was assessed through agar well diffusion assays, along with the determination of MIC and MBC. To evaluate biocompatibility, cytotoxicity analysis of the NPs was performed. The study also involved the preparation and characterisation of wound dressings impregnated with synthesised NPs, which were subsequently tested for antimicrobial activity. Finally, the in-vivo wound healing effects of these synthesised NPs-embedded dressings were examined through histopathological analysis and blood biochemical and haematological assessments, providing insights into their therapeutic potential for wound management.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Bioactive Compounds of Stingless Bee Honey

The growing recognition of phenolic compounds which is classified under bioactive compounds as potent antioxidants has stimulated significant interest in SBH. Hence, to elucidate the bioactive constituents of SBH, a comprehensive UHPLC-MS/Q-TOF analysis was performed. In this study, the results revealed a complex phytochemical profile comprising not only polyphenols but also organic acids and iridoid glucosides. This diverse array of compounds likely underpins the reported biological activities and potential health benefits of SBH. The chromatogram of the bioactive compounds found in SBH was given in Figure 4.1, and the list of the compounds were tabulated in Table 4.1.

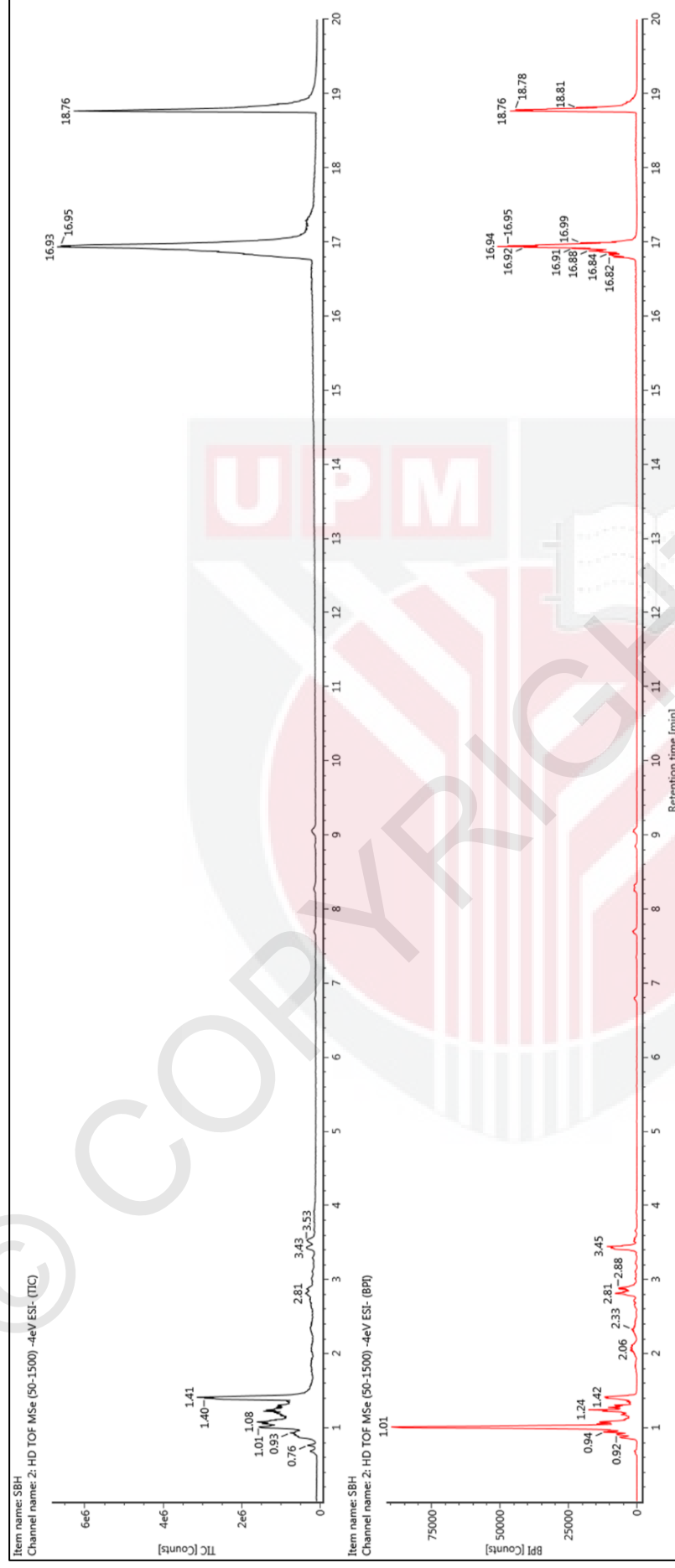


Figure 4.1: LCMSMS/Q-TOFF chromatogram for bioactive compounds in SBH

Table 4.1: Bioactive compounds in SBH

Component name	Formula	Neutral mass (Da)	Observed m/z	Mass error (ppm)	Observed RT (min)	Ionisation (+/-)
Polyphenolic Compounds						
<i>Phenolic Acids</i>						
1-O-Methyl-3,5-O-dicaffeoylquinic acid methyl ester	C ₂₇ H ₂₈ O ₁₂	544.15808	543.1528	3.7	0.98	-H
2,7,2',7',2''-Pentahydroxy-4,4',4'',7''-tetramethoxy-1,8,1',1''-triphenanthrene	C ₄₆ H ₃₄ O ₉	730.22028	729.2119	-1.5	1.38	-H
2''-O-Feruloylloesin	C ₂₉ H ₃₀ O ₁₂	570.17373	569.1663	-0.3	3.55	-H
Calycanthoside	C ₁₇ H ₂₀ O ₁₀	384.10565	383.0996	3.2	0.9	-H
Cnideoside A	C ₁₇ H ₂₀ O ₉	368.11073	367.105	4.1	0.88	-H
Courmaric acid	C ₉ H ₈ O ₂	148.05243	147.0445	-4.5	3.53	-H
3,4-Dihydroxy-6,7,3',4'-tetramethoxyflavone	C ₁₉ H ₂₀ O ₇	360.1209	359.1128	-2.3	1.08	-H
Dihydrokaempferol-5-O-β-D-glucopyranoside	C ₂₁ H ₂₂ O ₁₁	450.11621	449.1086	-0.7	7.98	-H
Epigallocatechin(4β,8)-gallocatechin	C ₃₀ H ₂₆ O ₁₄	610.13226	609.1237	-2.1	1.35	-H
Hydroxysafflor yellow A	C ₂₇ H ₃₂ O ₁₆	612.16903	611.1609	-1.3	1.43	-H
Iridin	C ₂₄ H ₂₆ O ₁₃	522.13734	521.1275	-4.9	2.72	-H
Kaempferitrin	C ₂₇ H ₃₀ O ₁₄	578.16356	577.1565	0.4	10.19	-H
Kansuinin C	C ₃₈ H ₄₂ O ₁₄	722.25746	721.2488	-1.8	1.41	-H
Odoratin-7-O-β-D-glucoside	C ₂₂ H ₂₄ O ₉	432.14203	431.1365	4	1.4	-H

Table 4.1: Continued

Component name	Formula	Neutral mass (Da)	Observed m/z	Mass error (ppm)	Observed RT (min)	Ionisation (+/-)
<i>Flavonoids (cont.)</i>						
Oroxin B	C ₂₇ H ₃₀ O ₁₅	594.15847	593.1523	1.9	1.05	-H
Safflomin C	C ₃₀ H ₃₀ O ₁₄	614.16356	613.1579	2.6	2.89	-H
Sanggenon B	C ₃₃ H ₃₀ O ₉	570.18898	569.1789	-4.9	1.42	-H
Silybin	C ₂₅ H ₂₂ O ₁₀	482.1213	481.1158	3.8	2.43	-H
Tetraacetylbrazililn	C ₂₄ H ₂₂ O ₉	454.12638	453.1211	4.3	1.43	-H
Zivulgarin	C ₂₈ H ₃₂ O ₁₅	608.17412	607.1693	4	1.4	-H
<i>Lignan</i>						
Eucommiol	C ₉ H ₁₆ O ₄	188.10486	187.0971	-2.8	11.79	-H
Sesamoside	C ₁₇ H ₂₄ O ₁₂	420.12678	419.1193	-0.6	3.53	-H
<i>Xanthones</i>						
Sibiricaxanthone A	C ₂₄ H ₂₆ O ₁₄	538.13226	537.1253	0.6	1.39	-H
<i>Other</i>						
Erigoster A	C ₂₇ H ₂₆ O ₁₃	558.13734	557.131	1.6	1.19	-H
Organic Acids						
3-Hydroxyphenyl-propionic acid	C ₉ H ₁₀ O ₃	166.06299	165.0553	-2.4	9.05	-H
5-Hydroxymethyl furoic acid	C ₆ H ₆ O ₄	142.02661	141.0188	-3.6	1	-H
Glucuronic acid	C ₆ H ₁₀ O ₇	194.04265	193.0348	-3.1	0.95	-H
Lactic acid	C ₃ H ₆ O ₃	90.03169	89.0242	-2.8	0.87	-H
Logaric acid (Loganic acid)	C ₁₆ H ₂₄ O ₁₀	376.13695	375.1288	-2.4	5.95	-H

Table 4.1: Continued

Component name	Formula	Neutral mass (Da)	Observed m/z	Mass error (ppm)	Observed RT (min)	Ionisation (+/-)
Organic acid (cont.)						
Methylsuccinic acid	C ₅ H ₈ O ₄	132.04226	131.0345	-3.5	1.41	-H
Phenylpropionic acid	C ₉ H ₁₁ NO ₂	165.07898	164.071	-4.2	3.52	-H
Trimethyl citrate	C ₉ H ₁₄ O ₇	234.07395	233.0659	-3.5	1.4	-H
Iridoid Glucoside						
6-Feruloyl catalpol	C ₂₅ H ₃₀ O ₁₂	522.17373	521.1667	0.5	0.97	-H
Asperuloside tetraacetate	C ₂₆ H ₃₀ O ₁₅	582.15847	581.1489	-4	3.03	-H
Meliadanoside B	C ₁₅ H ₂₀ O ₈	328.11582	327.1079	-2	7.69	-H
Mudanpioside J	C ₃₁ H ₃₄ O ₁₄	630.19486	629.1895	3	1.4	-H
Ningposide C	C ₁₇ H ₂₀ O ₈	352.11582	351.1096	3.1	0.87	-H
Picroside II	C ₂₃ H ₂₈ O ₁₃	512.15299	511.1456	-0.2	1.13	-H
Sesamoside	C ₁₇ H ₂₄ O ₁₂	420.12678	419.1193	-0.6	3.53	-H
Suffruticoside A	C ₂₇ H ₃₂ O ₁₆	612.16903	611.1593	-4	2.71	-H
Tectoruside	C ₂₁ H ₃₀ O ₁₃	490.16864	489.1613	-0.1	7.35	-H
Tubuloside D	C ₄₃ H ₅₄ O ₂₃	938.30559	937.301	2.9	1.4	-H
Tubuloside E	C ₃₁ H ₃₈ O ₁₅	650.22107	649.2161	3.6	1.23	-H
Others						
5R-5-Hydroxymethyl-2(5H)-furanone	C ₅ H ₆ O ₃	114.03169	113.0241	-2.9	0.87	-H
Desmodimine	C ₁₂ H ₁₅ NO ₄	237.10011	236.0921	-3.3	3.91	-H
Matol-β-D-glucopyranoside	C ₁₂ H ₁₆ O ₉	304.07943	303.0717	-1.5	1.4	-H
Meso-inositol	C ₆ H ₁₂ O ₆	180.06339	179.0555	-3.3	0.97	-H
Mirificin	C ₂₆ H ₂₈ O ₁₃	548.15299	547.1449	-1.4	0.88	-H

Table 4.1: Continued

Component name	Formula	Neutral mass (Da)	Observed m/z	Mass error (ppm)	Observed RT (min)	Ionisation (+/-)
Others (Cont.)						
Neocuscutoside C	C ₃₂ H ₃₈ O ₁₇	694.2109	693.2063	3.9	1.4	-H
Nuezhenidic acid	C ₁₇ H ₂₄ O ₁₄	452.11661	451.1076	-3.8	1.11	-H
Scroside A	C ₃₇ H ₅₀ O ₂₁	830.28446	829.2738	-4.1	1.1	-H
Viscumneoside V	C ₃₂ H ₄₀ O ₁₉	728.21638	727.2114	3.2	1.24	-H

From Table 4.1, polyphenolics are identified as the major bioactive compounds in the SBH used for this study. Four main subclasses of polyphenolics were found from this study including phenolic acids, flavonoids, lignans, and xanthenes. From these results, six phenolic acids, fourteen flavonoids, two lignans, and one xanthone were identified within the SBH sample. Flavonoids were the most abundant phenolic compounds detected. Aligning with previous research conducted by Ali et al. (2021), flavonoids are recognised as pivotal secondary metabolites with extensive applications in nutraceuticals, functional foods, and pharmaceuticals. Their potent antioxidant, anti-mutagenic, anti-carcinogenic, and anti-inflammatory properties underscore their significance.

Moreover, polyphenolic compounds are well-established for their antioxidant, antimicrobial, and anti-inflammatory attributes (Pauliuc et al., 2020). Chettri and Kumari (2020), stated that SBH contains certain phenolic compounds capable of treating infections caused by pathogenic microorganisms, including multidrug-resistant strains such as *S. aureus*, *E. coli*, *Proteus vulgaris*, *Shigella sonnei*, and *Klebsiella* species. Additionally, penanthrene, one of the phenolic acid compounds have been studied for their biological activities including their potential toxicity, antimicrobial, anti-inflammatory, and antioxidant effect (Tóth et al., 2018). Beyond antimicrobial actions, polyphenols exhibit diverse biological functions, such as enzyme modulation, anti-proliferative effects, and antioxidant activity, crucial for counteracting oxidative stress induced by reactive oxygen species (ROS) (Ali et al., 2021).

Organic acids are another type of bioactive compound found in SBH. Despite being present in low concentrations, they significantly contribute to the organoleptic, physical, and chemical properties of SBH. This study identified eight organic acids: 3-Hydroxyphenyl-propionic acid, 5-Hydroxymethyl furoic acid, glucuronic acid, lactic acid, loganic (or loganic) acid, methylsuccinic acid, phenylpropionic acid, and trimethyl citrate. This finding contrasts with the previous report by Sujanto et al. (2021), which identified only four types of organic acids including gluconic, lactic, acetic, and citric acids in SBH samples from *G. thoracica* and *H. itama*.

Meanwhile, iridoid glucoside are a type of bioactive compound, which are monoterpenoids with a cyclopentanopyran ring system. As glucosides, they are bound to one or more sugar molecules (typically glucose). Iridoid glucosides exhibit various biological activities, including anti-inflammatory, antioxidant, as well as antimicrobial (Chahardoli et al., 2020). There are 11 iridoid glucoside were detected in this study and they are 6-Feruloyl catalpol, asperuloside tetraacetate, meliadanoside B, mudanpioside J, ningposide C, picroside II, sesamoside, suffruticoside A, tectoroside, tubuloside D, and tubuloside E.

As presented in Section 2.2 (Table 2.2), The differences in the bioactive compounds of SBH are significantly influenced by various factors, particularly the botanical sources from which the nectar is derived, as well as the specific pollen and nectar collected by the bees themselves (Pauliuc et al., 2020). These differences in source material contribute to the unique chemical

composition of each batch of honey. Consequently, the variations in bioactive compounds may not only affect the nutritional profile but also the potential health benefits associated with each type of honey. Furthermore, this variability can impact their properties for further applications in fields such as nutrition, medicine, and cosmetics. Understanding these influences is crucial for harnessing the full potential of SBH in various industries, as well as for informing consumers about the diverse benefits that different types of honey can offer.

Rather than focusing on their therapeutic benefits, this study also identifies the bioactive compounds in SBH that could be utilised for NP synthesis. The bioactive compounds acted as reducing and capping agents, facilitating the synthesis and stabilisation of the NPs (Habsi et al., 2019; Zahoor et al., 2021). The relation between the bioactive compounds found in SBH and the agglomeration of NPs formed is largely dependent on the type of bioactive compounds present. The presence of certain polysaccharides, proteins, and other organic molecules can affect the stability and dispersity of the NPs, leading to their aggregation. The exact nature of this interaction and the influence of specific bioactive compounds on the stability of NPs synthesised using SBH are still being studied and understood. However, it is believed that the presence of certain hydrophilic or hydrophobic moieties in the bioactive compounds can affect the surface energy and charge of the NPs, leading to their aggregation or stability.

4.2 Synthesis and Characterisation of Silver, Zinc Oxide, and Bimetallic Silver/Zinc Oxide Nanoparticles using Stingless Bee Honey.

The synthesis of NPs using honey, particularly SBH, involves intricate mechanisms that leverage the diverse components present in honey for the formation, stability, and unique properties of the NPs. The primary reducing agents in honey are glucose and fructose, which make it particularly suitable for green synthesis methods (Hosny et al., 2017). These natural sugars play a crucial role in reducing metal ions, such as Cu^{2+} to Cu_2O , which can be observed in Benedict's solution, and Ag^+ to Ag^0 , as seen with Tollens' reagent, through specific oxidation reactions (Bonsignore et al., 2021). In an alkaline environment, glucose and fructose undergo oxidation, producing gluconic acid. This process is further supported by the potent reducing properties of vitamin C, which is also present in honey (Balasooriya et al., 2017).

Moreover, proteins and enzymes found in honey significantly contribute to the synthesis of NPs. Notably, glucose oxidase facilitates the conversion of glucose into gluconic acid and hydrogen peroxide, which subsequently reacts with metal ions to produce metal oxides. Other enzymes, such as catalase and peroxidase, further decompose hydrogen peroxide, enhancing the formation of metal oxides in the process. Additionally, the proteins in honey act as effective capping agents, preventing NP agglomeration and oxidation while simultaneously influencing their size, shape, and overall stability (Haiza et al., 2013).

SBH, as mentioned in Section 4.1, being rich in bioactive compounds such as sugars, amino acids, phenolics, and organic acids, plays a dual role as both a reducing and stabilising agent in the synthesis of NPs (Biluca et al., 2020; Maringgal et al., 2019). The electron-donating properties of these compounds facilitate the reduction of metal ions to their metallic forms, thereby playing a vital role in NP synthesis. The effectiveness of SBH in this synthesis is closely linked to the bioactive content and its concentration, both of which can be significantly influenced by the specific source of the honey and its processing methods (Haron et al., 2022; Sharin et al., 2021).

In the synthesis of Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs, the use of SBH proves to be highly effective, functioning as both a reducing and stabilising agent. The carbonyl groups present in the various sugars found in honey are capable of reducing metal ions, such as Ag^+ and Zn^{2+} , to their elemental states. Meanwhile, the alcohol groups contribute to the stabilisation of the resulting NPs by forming protective layers through various means, including hydrogen bonding, electrostatic interactions, or van der Waals forces (Saravanan et al., 2021). The acidic pH and the hydrogen peroxide content present in honey significantly enhance the reduction of Ag^+ , even under otherwise alkaline conditions. Notably, studies have indicated that the NPs synthesised using honey exhibit similar particle size trends to those produced using individual sugars, with higher concentrations of glucose leading to smaller particle sizes (Balasooriya et al., 2017).

The overall synthesis process initiates with the dissociation of metal salts, specifically AgNO_3 and $(\text{Zn}(\text{NO}_3)_2)$, into their respective cations: Ag^+ and Zn^{2+} . This is followed by the formation of complexes with biomolecules sourced from SBH, as portrayed in Figure 4.2. The subsequent addition of NaOH facilitates the creation of metal hydroxide-SBH complexes. In this alkaline environment, the glucose ring is opened through the extraction of β -protons from the oxygen in the sugar ring, enabling the metal ions to oxidise glucose into gluconic acid.

According to Habsi et al. (2019), honey exhibits a higher abundance of negatively charged functional groups, which enhances its ability to bind effectively and reduce metal salts into NPs even in alkaline conditions. Conversely, under acidic conditions, the aggregation of bacterial cells within the honey promotes better dispersion of the particles. After continuous stirring of the reaction mixture at a controlled temperature of 80°C for a duration of 2 hours, the resulting precipitate is then dried at 60°C for 24 hours to yield the final NPs. Throughout this synthesis process, SBH plays a crucial role in effectively reducing AgNO_3 to generate Ag-NPs, $\text{Zn}(\text{NO}_3)_2$ to produce ZnO-NPs, and a combination of both salts to create bimetallic Ag/ZnO-NPs, ultimately showcasing the versatility and effectiveness of using natural resources in nanoparticle synthesis.

A similar study conducted in 2019 by Ismail et al. proposed a promising mechanism for synthesising copper NPs (Cu-NPs) using honey. In this process, copper ions (Cu^{2+}) from copper nitrate interact with the negatively charged hydroxyl groups of fructose and glucose in honey, forming

[Cu(Honey)]²⁺ complexes. Upon adding NaOH, these complexes are converted into [Cu(OH)₂/honey], which subsequently produce Cu-NPs with the addition of ascorbic acid and ultrasonic irradiation, significantly reducing the reaction time. The positive charge on the Cu-NPs surface forms electrostatic bonds with the negatively charged hydroxyl groups in fructose and glucose, ensuring uniform particle size distribution. Additionally, the polyhydroxy groups in glucose and fructose prevent the formation of large agglomerates.

This process underscores the dual role of SBH in NPs synthesis. The first role is facilitating the reduction of metal ions and stabilising the resulting NPs to prevent aggregation, and the second one is, the stability and characteristics of the synthesised NPs are influenced by the nature of the bioactive compounds in SBH, which interact with the NPs surfaces in various ways to ensure their effective stabilisation.

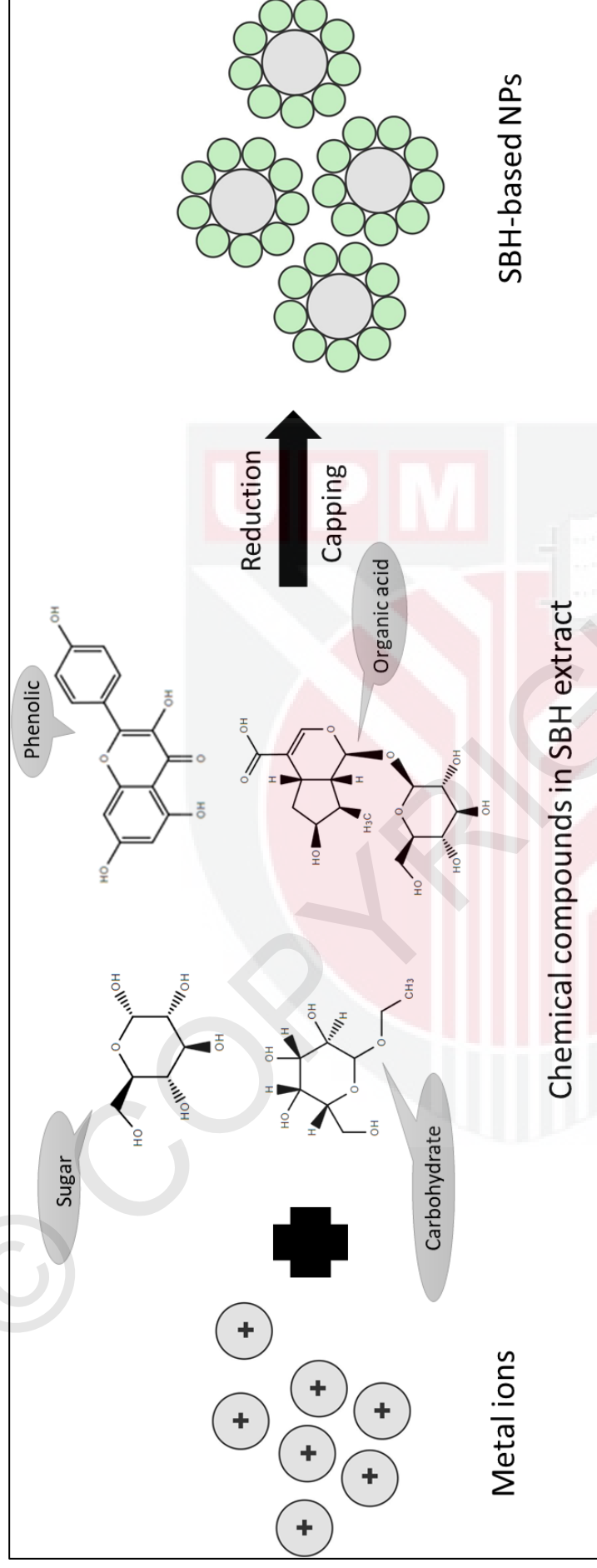


Figure 4.2: Suggested mechanism that involves in the preparation of synthesised NPs.

4.2.1 Formation of Silver, Zinc Oxide and Bimetallic Silver/Zinc Oxide Nanoparticles using Stingless Bee Honey.

A visual colourimetric analysis was conducted as an initial assessment to monitor the formation of NPs, with distinct colour changes indicating the reduction of metal ions (Figure 4.3). Initially, the solutions transitioned from colourless to yellow, followed by a brown colouration and subsequent precipitation within five minutes, confirming the successful synthesis of NPs (Vinay & Chandrasekhar, 2021). The addition of SBH to an AgNO_3 solution at pH 11 resulted in a dark brown colour, signifying the formation of Ag-NPs. This notable change is attributed to the Surface Plasmon Resonance (SPR) phenomenon, where the collective oscillation of conduction electrons in Ag-NPs interacts with light, leading to absorption and scattering that produce the characteristic brown hue. The intensity of this colour is significantly influenced by NP size, shape, and concentration, as smaller particles exhibit more intense colouration due to a higher surface-to-volume ratio and pronounced SPR effects (Khalir et al., 2020).

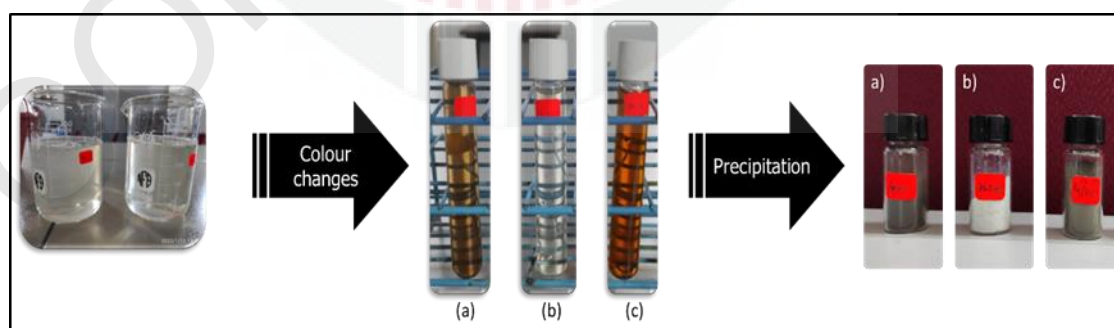


Figure 4.3: Formation of synthesised NPs; a) Ag-NPs, b) ZnO-NPs, and c) bimetallic Ag/ZnO-NPs.

Similarly, when SBH was added to $\text{Zn}(\text{NO}_3)_2$ at the same pH, the solution turned yellow, indicating the reduction of Zn^{2+} ions to ZnO-NPs. The yellow hue reflects the quantum confinement effects of ZnO-NPs, where the reduction in particle size alters the electronic band structure, causing light absorption in the UV-visible range (Al-Asady et al., 2020). The formation of bimetallic Ag/ZnO-NPs produced an intermediate colour between yellow and dark brown, suggesting the successful incorporation of both metals. This mixed colour results from the combined optical properties of Ag and ZnO, where the SPR band of Ag and the electronic transitions of ZnO influence the final appearance (Kyomuhimbo et al., 2019).

These colourimetric changes provide valuable insights into the formation and properties of the synthesised NPs. However, variations in colour intensity and final appearance suggest differences in particle size distribution, aggregation tendencies, and overall stability. While the results align with previous studies demonstrating the effectiveness of SBH as a bioreductant, discrepancies arise in NP stability. Unlike studies that reported improved dispersion with additional stabilising agents (Vinay & Chandrasekhar, 2021), this study indicates potential aggregation due to incomplete capping, which could reduce surface area and reactivity (Chopra et al., 2022). This highlights the need for further optimisation of synthesis parameters, including pH, SBH concentration, and reaction conditions, to enhance stability. Additionally, the observed colour shift in bimetallic Ag/ZnO-NPs aligns with prior research on hybrid metal systems (Kyomuhimbo et al., 2019), however, variations in hue suggest differences in particle interactions and distribution.

4.2.2 Characterisation of Silver, Zinc Oxide, and Bimetallic Silver/Zinc Oxide Nanoparticles

4.2.2.1 Ultraviolet-visible spectroscopy

The absorption spectra of metal and metal oxide NPs typically exhibit prominent peaks that correspond to SPR, a phenomenon that arises from the collective oscillation of conduction electrons within the particles. The specific position and width of these SPR peaks are significantly influenced by several factors, including particle size, shape, and the surrounding dielectric environment (Khalir et al., 2020). In this study, we employed UV-Vis spectrophotometry to confirm the successful formation of Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs by analysing their absorption spectra across the 280 to 600 nm wavelength range (Figure 4.4).

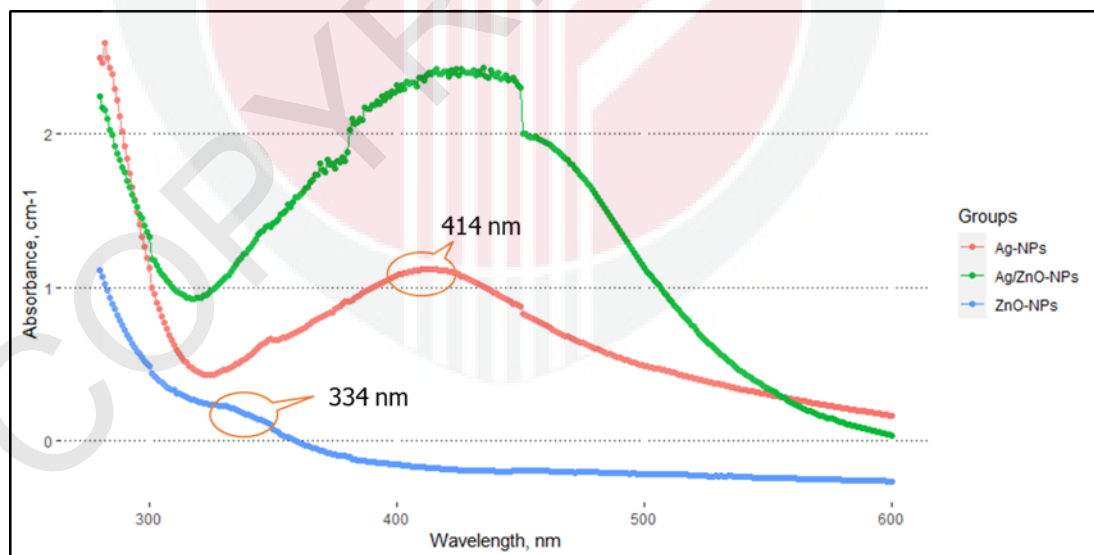


Figure 4.4: UV-Vis spectra for the synthesis of synthesised NPs.

For the Ag-NPs, a distinct SPR peak was observed at 414 nm, corroborating their successful synthesis. This result aligns well with the findings of Jyoti et al. (2016), who documented a similar SPR peak at 414 nm, indicating that the synthesised particles have reached nanoscale dimensions and predominantly exhibit a spherical shape. Furthermore, studies conducted by Bhubhanil et al. (2021) and Pascu et al. (2021) reported SPR peaks ranging from 403 to 415 nm, depending on various synthesis conditions and pH levels, thereby reinforcing the consistency of our findings. However, it is important to note that some variations exist in the literature, for instance, a peak at 429 nm was reported by Afzal et al. (2023). Such discrepancies may be attributed to differences in synthesis methods, the types of reducing agents employed, and variations in the surrounding medium, all of which can significantly influence the dielectric environment and electron density around the NPs. The sharpness and broadness of the SPR peak observed in this study suggests a size distribution, which is crucial for ensuring consistent optical and functional properties across applications.

In relation to the ZnO-NPs, an absorption peak was detected at 334 nm, indicating strong absorption within the UV region. This finding aligns with the observations made by Al-Asady et al. (2020), who reported that ZnO-NPs typically exhibit absorption within the 200 to 400 nm range. However, contrasting results were documented by Rasha et al. (2021), who reported peaks for ZnO-NPs between 360 and 380 nm when synthesised using *Acacia nilota*. Similarly, Elhabal et al. (2024) observed a peak at 390 nm for ZnO-NPs synthesised with *Althae officinalis* extract. The slight blue shift noted in this

study compared to bulk ZnO suggests the presence of a quantum confinement effect, wherein reduced particle size alters the electronic band structure, leading to a shift in the absorption band. Additionally, the presence of SBH as a stabilising agent may have influenced the surface states of the NPs, consequently affecting their optical properties. These differences underscore the significant impact of varying synthesis conditions and bio-reducing agents on the final properties of ZnO-NPs.

For bimetallic Ag/ZnO-NPs, a broad SPR band was observed between 380 and 460 nm, reflecting the electromagnetic coupling between Ag and ZnO within the hybrid structure. This broadening and shift in the SPR peak suggest that there are strong interactions between the two components, leading to increased charge density and an altered dielectric environment. Similar effects were reported by Kyomuhimbo et al. (2019), who noted that in-phase interactions among adjacent NPs contribute significantly to this spectral broadening. The shift in the SPR band compared to monometallic Ag-NPs indicates that the combined plasmonic and electronic properties of Ag and ZnO have a profound influence on the overall optical response of the hybrid material. However, variations exist in the literature, such as the 399 nm peak observed for Ag/ZnO-NPs by Afzal et al. (2023), likely due to differences in NP size, synthesis conditions, and the nature of the capping agents used during the preparation process. As highlighted by Joudeh & Linke (2022), these factors play a crucial role in determining the absorption peak position by affecting not just electron density but also particle morphology and overall stability.

This stability suggests that the use of SBH effectively acts as a stabilising agent, preventing aggregation and preserving the optical properties of the NPs over time. The presence of bioactive compounds such as flavonoids and phenolic acids in SBH likely plays a key role in capping the NPs and enhancing their stability in solution.

4.2.2.2 Field Emission Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy

The morphology and size distribution of the synthesised NPs were meticulously analysed using FESEM, while their elemental composition was confirmed through EDX. The FESEM images revealed that the NPs predominantly exhibited a spherical morphology, although some hexagonal structures were also observed, as illustrated in Figure 4.5. This variation in shape is likely influenced by the diverse biomolecules present in SBH, including flavonoids, glucosides, phenolic acids, and tannins, which play a critical role in the biomineralisation process, as noted by Elhabal et al. (2024). The synthesis conditions, particularly the type and concentration of bioactive compounds in SBH, significantly influenced the final morphology of the NPs. The average sizes of the SBH synthesised Ag-NPs, ZnO-NPs, and Ag/ZnO-NPs were found to be 30.09 ± 12.36 nm, 19.33 ± 4.21 nm, and 33.05 ± 10.63 nm, respectively. These findings indicate that the synthesis parameters were effective in controlling NP size, a crucial factor in determining their physicochemical properties and functional applications in various fields, including medicine and environmental remediation.

The elemental composition analysis via EDX corroborated the FESEM results, confirming the presence of Ag in Ag-NPs, Zn in ZnO-NPs, and both Ag and Zn in the bimetallic Ag/ZnO-NPs. Specifically, Ag-NPs exhibited a characteristic Ag L line peak at 3.2 keV, while ZnO-NPs displayed Zn L, Zn K α , and Zn K β peaks at 1, 8.6, and 9.5 keV, respectively. The presence of additional elements such as Cl, Al, F, Mg, C, and O across all samples was likely due to SBH components and potential environmental contamination, while copper peaks originated from the FESEM grid itself. The detection of C, Na, and Al in the EDX spectra strongly suggests the degradation of capping agents, such as polysaccharides, proteins, amino acids, and sugars, during the EDX analysis, as reported by El-Belely et al. (2021). The confirmation of both Ag and Zn peaks in the bimetallic Ag/ZnO-NPs further validated their successful formation, supporting the efficiency and efficacy of the green synthesis approach, which is not only environmentally friendly but also economically viable for producing high-quality nanoparticles. This research highlights the potential of using natural resources like SBH in nanotechnology applications, paving the way for future studies aimed at exploring the full range of possibilities that these synthesised NPs offer.

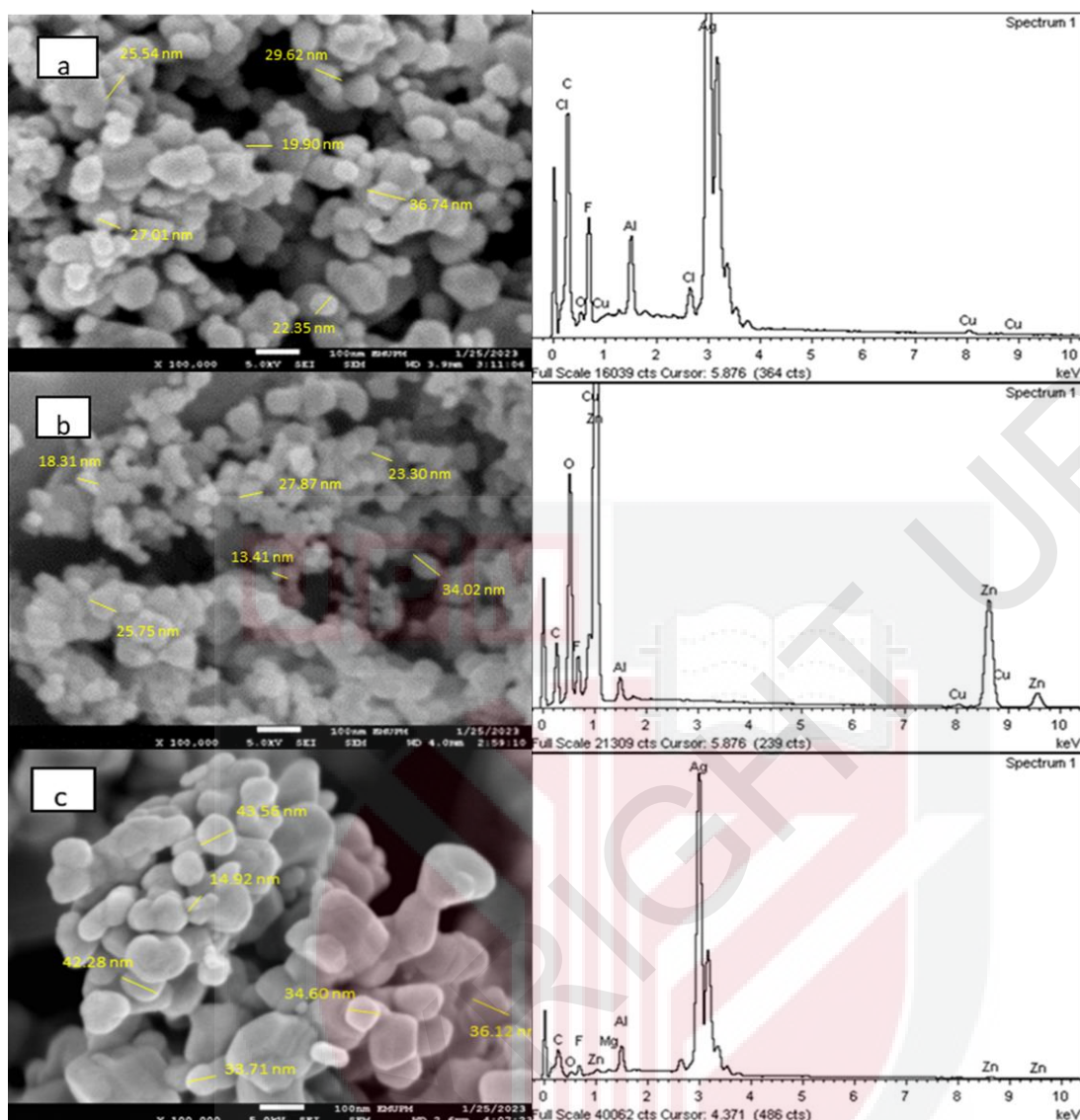


Figure 4.5: FESEM-EDX micrograph for (a) Ag-NPs, (b) ZnO-NPs, and (c) bimetallic Ag/ZnO-NPs.

In this study, the synthesis was conducted under alkaline conditions (pH 11), a factor that significantly influenced NP morphology and size. Ribut et al. (2019) reported that pH plays a crucial role in NP formation, with Pascu et al. (2021) noting that at pH levels below 10, NP distribution is uneven with lower yields. Conversely, at pH levels above 10, a more uniform distribution and higher NP concentrations are achieved. The influence of pH on NP synthesis is well-documented, as it affects the charge of phytochemicals, subsequently

altering metal ion reduction and complexation processes. Under alkaline conditions, hydrolysis and complexation reactions are enhanced, leading to more controlled NP growth with well-defined sizes and shapes (Jorge de Souza et al., 2019). Arshad et al. (2021) further emphasised that higher pH levels accelerate NP synthesis due to increased electron availability, which facilitates a more efficient reduction process. Additionally, pH affects the local surface characteristics of NPs through protonation and deprotonation processes during nucleation and growth, influencing their stability and preventing aggregation (Patil & Chandrasekaran, 2020). The findings of this study align with these reports, reinforcing the critical role of pH in optimising NP characteristics for various applications.

Besides pH, reaction temperature also played a significant role in NP synthesis. The process was conducted at 80°C for 2 hours, a parameter chosen to enhance enzymatic activity and reaction kinetics. Bala & Rani (2020) noted that maintaining an optimal temperature ensures sufficient time for NP growth, allowing them to reach the desired morphology without excessive reaction times, which can lead to undesired outcomes. Furthermore, an overnight incubation period further improved NP formation, leading to larger yields, better size control, and enhanced stability (Chopra et al., 2022). This highlights the importance of carefully balancing temperature and reaction duration to achieve high-quality NPs, as both factors are integral to the overall synthesis process.

To further prevent NP aggregation, a low drying temperature was employed during the final stage of synthesis. High drying temperatures can cause rapid evaporation, leading to NP aggregation due to close particle proximity. Chadha et al. (2022) reported that maintaining a low drying temperature preserves NP morphology and prevents the loss of essential surface characteristics. The careful selection of synthesis parameters; pH, temperature, and drying conditions was crucial in obtaining well-dispersed, stable NPs suitable for various applications.

4.2.2.3 Transmission Electron Microscopy

The TEM micrographs (Figure 4.6) provided compelling evidence of a thin protective layer enveloping the synthesised NPs. This observation confirms the successful coating of the NPs with bioactive compounds derived from SBH. The synthesis process of Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs utilising SBH is characterised by a simultaneous reduction and stabilisation mechanism, where SBH serves a dual purpose as both a reducing and capping agent. This dual functionality is essential for not only the effective formation of NPs but also ensuring their long-term stability and integrity in various environments. As highlighted by Chugh et al. (2021), biologically synthesised NPs are typically capped by a diverse range of biomolecules, including alkaloids, phenolic compounds, terpenoids, enzymes, co-enzymes, proteins, polysaccharides, and lipids. In the specific case of SBH synthesis, polyphenols, proteins, and sugars are particularly influential in facilitating the metal ion reduction process and stabilising the resultant NPs. The interaction

between the negatively charged functional groups present in these bioactive molecules and the positively charged surfaces of the NPs generates electrostatic repulsion. This phenomenon effectively prevents aggregation and promotes controlled growth of the NPs, as noted by Khalir et al. (2020).

The TEM analysis further demonstrated that the protective bioactive layer significantly contributed to the uniform size distribution of the synthesised NPs, underscoring the effectiveness of SBH compounds in regulating NP morphology. However, a slight degree of agglomeration was observed, particularly among the bimetallic Ag/ZnO-NPs. This agglomeration phenomenon can be attributed to the concentration of reactants used during the synthesis process, specifically the metal precursors and reducing agents. High concentrations of reactants result in an increased number of reducing agents binding to the surface of the preformed nuclei, thereby accelerating secondary reduction processes. Consequently, the rapid growth of NPs can lead to larger particle sizes and potential aggregation. Patil & Chandrasekaran (2020) previously reported that an excess of reducing agents can induce bridging interactions between NPs, facilitating their agglomeration.

When comparing these results with existing research, it is evident that the concentration of bioactive compounds plays a significant role in influencing NP stability. A notable study by Sirelkhatim et al. (2015) emphasised that carefully controlled synthesis parameters, particularly reactant concentrations, are crucial for preventing unwanted NP agglomeration. Similarly, Khan et al. (2021) observed that excessive interactions among biomolecules could lead

to NP clustering, thereby reducing their surface area and adversely affecting their functionality in various biomedical applications. In light of these observations, the agglomeration noted in the bimetallic Ag/ZnO-NPs suggests that further optimisation of precursor concentrations and reaction conditions is necessary to achieve better dispersion and stability of the NPs.

Additionally, the protective bioactive layer observed in the TEM images is a critical factor influencing the physicochemical properties of the synthesised NPs. Previous studies have demonstrated that biologically derived capping agents enhance NP biocompatibility, a vital criterion for applications in the biomedical field (Zhang et al., 2020). The presence of natural capping agents within SBH synthesised NPs may contribute to their potential antimicrobial and wound-healing properties, aligning with findings reported by Abd Elkodous et al. (2019).

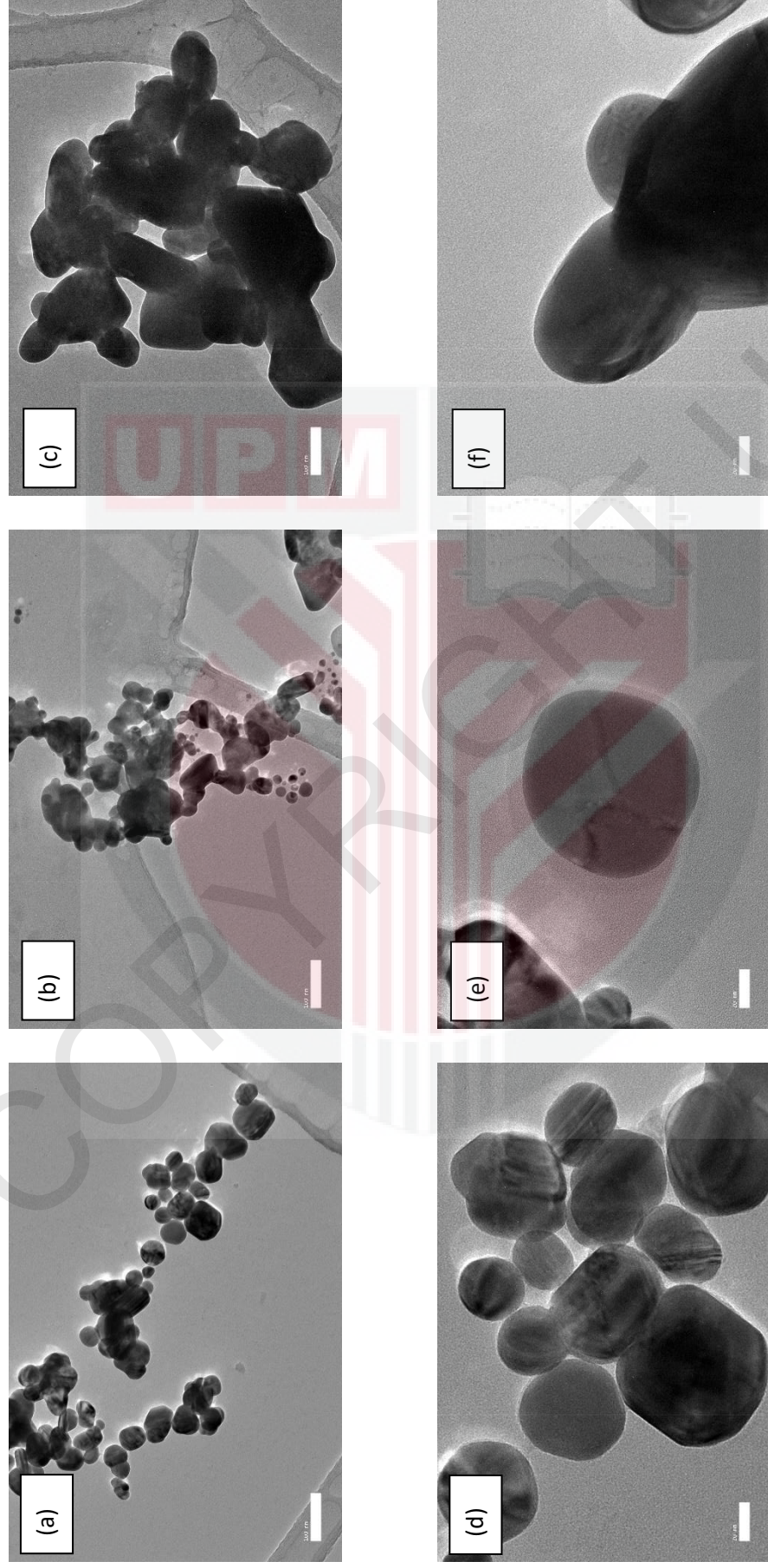


Figure 4.6: TEM micrograph of Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs at 25000x (a, b, & c) and 100000x (d, e, & f) magnification.

4.2.2.4 Fourier Transform Infrared Spectroscopy

FTIR spectroscopy was conducted to identify the functional groups responsible for the reduction and stabilisation of the synthesised Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs, complementing the findings from TEM analysis (Section 4.2.2.3). The FTIR spectra revealed distinct absorption bands, confirming the presence of key functional groups, as illustrated in Figure 4.7 (a-c) and summarised in Table 4.2. These functional groups, originating from the bioactive compounds in SBH, play a crucial role in NP formation and stabilisation, supporting their potential biomedical applications.

A broad absorption band centered around 3000 cm^{-1} was observed, indicative of O-H stretching vibrations associated with alcohols and phenols. This aligns with Yousaf et al. (2020), who reported a similar peak at 3357 cm^{-1} , attributing it to polyphenolic hydroxyl groups. Kozłowicz et al. (2020) further suggested that this spectral region encompasses contributions from carbohydrates, water, organic acids, and amino acids, all of which are naturally present in honey. The presence of these hydroxyl-containing compounds suggests their active involvement in metal ion reduction, confirming honey's dual role as both a reducing and capping agent. The interaction of these functional groups with the NPs surface likely contributes to their colloidal stability, preventing aggregation and promoting uniform dispersion.

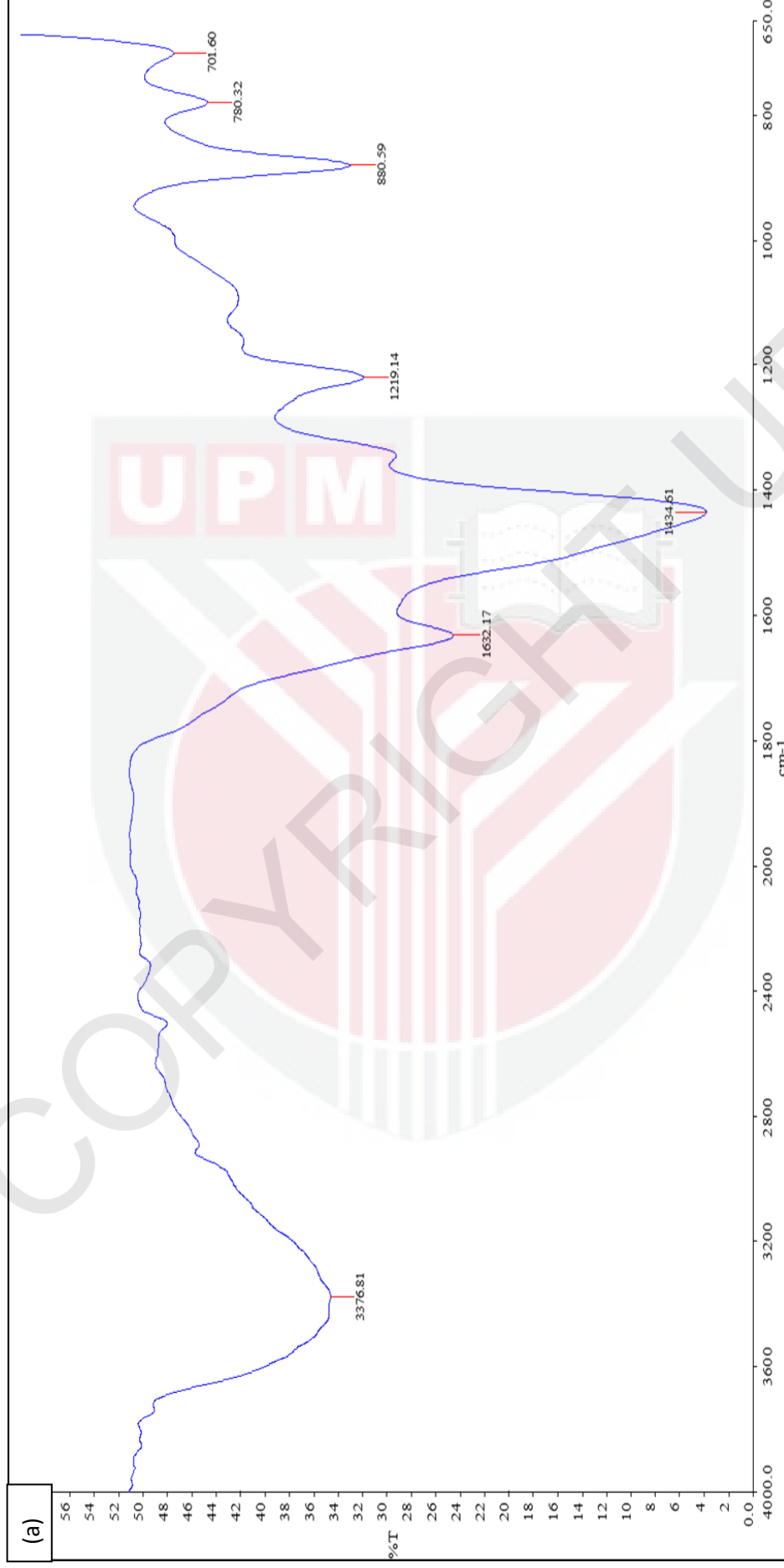


Figure 4.7: (a) FTIR spectrum of Ag-NPs.

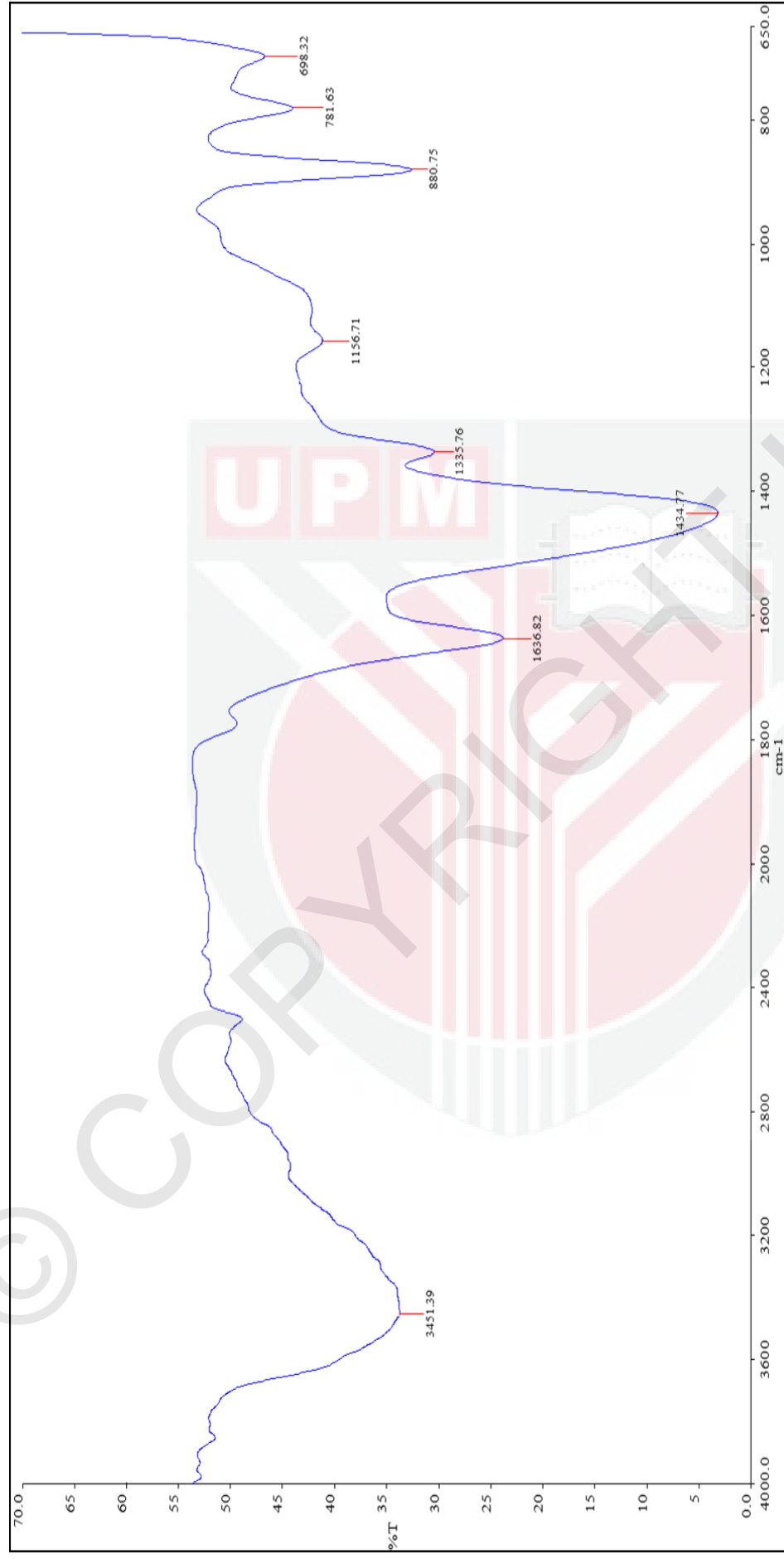


Figure 4.7: (b) FTIR spectrum of ZnO-NPs

(c)

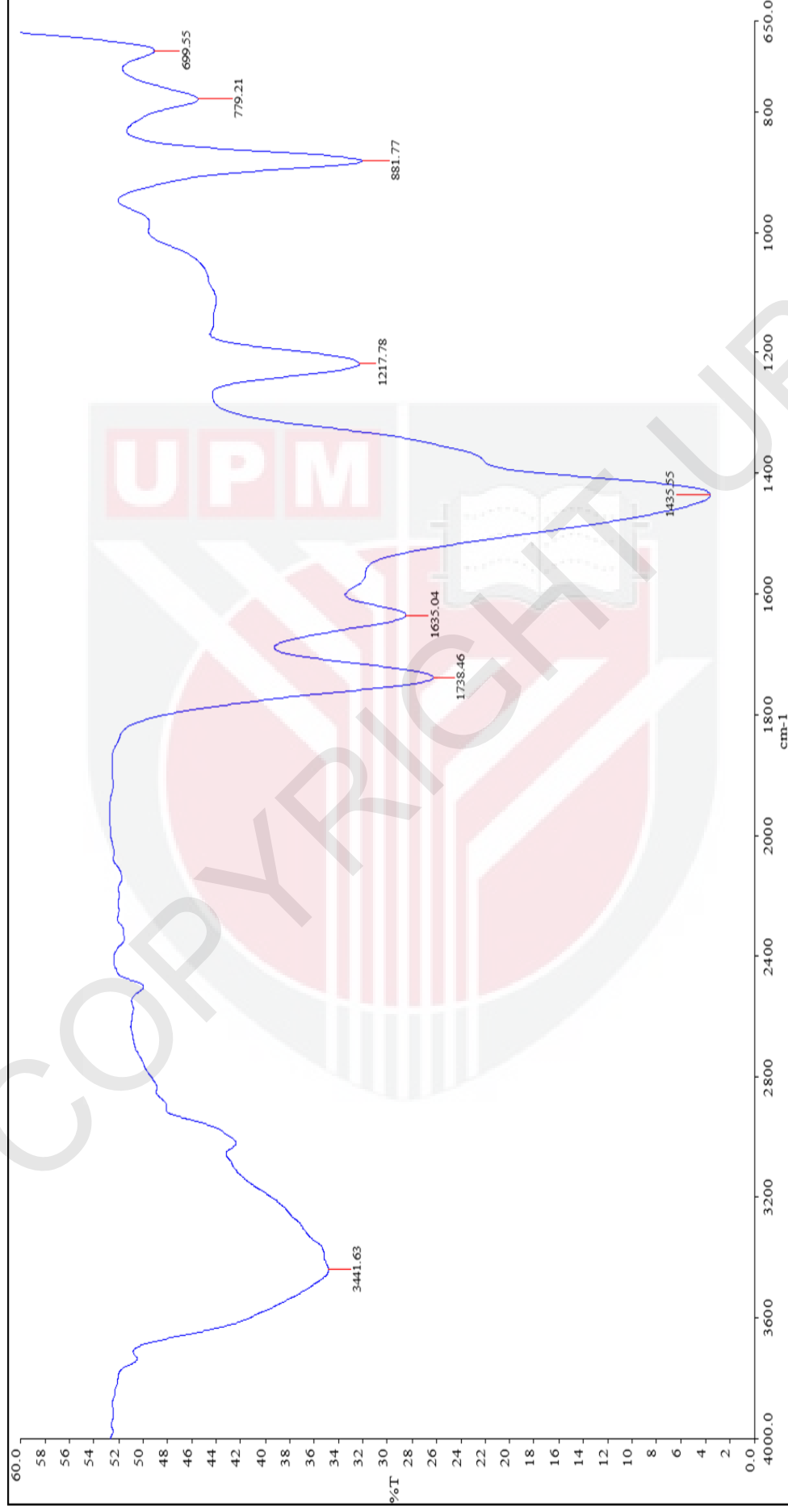


Figure 4.7: (c) FTIR spectrum of bimetallic Ag/ZnO-NPs.

Table 4.2: FTIR bands for Synthesised Nanoparticles.

SBH-based Nanoparticles	Wavelength, cm⁻¹	Functional Groups
Ag-NPs	3376.81	Strong, broad O-H stretching, intermolecular bonded alcohol and phenol
	1632.17	Medium N-H bending of amine
	1434.61	C-O-H protein
	1219.14	Medium C-N stretching (amide III)
	880.59	Strong C=C bending
	780.32 & 701.60	Medium C=C bending
ZnO-NPs	3451.39	Strong, broad O-H stretching, intermolecular bonded alcohol and phenol
	1636.82	Medium N-H bending of amine
	1434.77	C-O-H protein
	1335.76	O-H bending alcohol
	1156.71	C-O stretching of protein
	880.75	Strong C=C bending
	781.63 & 698.32	Medium C=C bending
Bimetallic Ag/ZnO-NPs	3441.63	Strong, broad O-H stretching, intermolecular bonded alcohol and phenol
	1738.46	Strong C=O stretching, carboxylic acid group of the gluconic acid
	1635.04	Medium N-H bending of amine
	1435.55	C-O-H protein
	1217.78	Medium C-N stretching (amide III)
	881.77	Strong C=C bending
	779.21 & 699.55	Medium C=C bending

The FTIR spectra also displayed a strong C=O stretching band in the 1600-1700 cm^{-1} range, characteristic of carboxylic acid groups. Agudelo et al. (2018) associated this band with gluconic acid, while Elhabal et al. (2024) attributed it to flavonoids in honey, both of which enhance NP stability through electrostatic interactions. Similarly, Liang et al. (2020) observed an N-H bending peak at 1600 cm^{-1} , supporting the role of amine groups in stabilisation. The presence of a C-O-H protein stretching band at 1400 cm^{-1} further corroborates the findings of Norouzi et al. (2021), indicating the contribution of honey-derived proteins to NP stabilisation. These proteins likely interact with metal surfaces through hydrogen bonding and coordinate interactions, forming a protective layer that prevents particle agglomeration.

Additionally, the presence of an amide III band at 1200 cm^{-1} (C-N stretching) and a C-O stretching peak at 1150 cm^{-1} further confirms the involvement of proteins in NP stabilisation. Ismail et al. (2019) suggested that these peaks arise from honey proteins' C-O stretching vibrations, emphasising their role as capping agents. Peaks within the 1400-1300 cm^{-1} and 1000-1100 cm^{-1} regions were attributed to C-H deformations of aliphatic compounds and C-O stretching of alcohols or esters, respectively. This aligns with Se et al. (2018), who linked the 1500-1800 cm^{-1} region to monosaccharides and disaccharides, further supporting the hypothesis that sugars contribute to NP stabilisation.

The presence of sugar-derived peaks, particularly at 1054 cm^{-1} (fructose) and 800-1100 cm^{-1} (glucose), suggests their potential role in NP formation. Zhang et al. (2020) reported that sugars act as stabilising agents by forming a

protective layer around NPs, preventing agglomeration. However, the TEM micrographs (Section 4.2.2.3) revealed some degree of agglomeration in bimetallic Ag/ZnO-NPs, suggesting that the interaction between reducing and capping agents may need further optimisation. Khan et al. (2021) noted that excessive biomolecule interactions can lead to NP clustering, reducing their surface area and limiting their bioactivity. This highlights the need to fine-tune the synthesis process to balance reduction and stabilisation, thereby achieving optimal NP dispersion.

The FTIR results also confirm the complex interplay of organic compounds in SBH during NP synthesis, reinforcing prior studies on honey-mediated NP formation. The combination of polyphenols, proteins, and sugars provides a multifunctional approach to NP stabilisation, as supported by Hosny et al. (2017), who demonstrated that honey proteins, particularly those containing free amine and carboxylate groups, play a major role in metal NP capping. Similarly, Sirelkhatim et al. (2015) emphasised that the presence of these functional groups enhances NP dispersibility, a crucial factor for biomedical applications.

However, despite the strong stabilisation effects of honey-derived compounds, the slight agglomeration observed in bimetallic Ag/ZnO-NPs indicates a need for optimisation. This finding aligns with Patil & Chandrasekaran (2020), who reported that excessive reducing agent concentrations could lead to uncontrolled NP growth, promoting agglomeration. Future studies should investigate the influence of pH, precursor concentration, and reaction kinetics

to refine NP synthesis conditions. Additionally, incorporating surfactants or additional stabilising agents could further enhance NP stability, as suggested by Ismail et al. (2019).

Furthermore, the observed functional groups suggest potential applications beyond wound healing. The presence of hydroxyl, carboxyl, and amine groups enhances the biocompatibility of the synthesised NPs, making them suitable for drug delivery, antimicrobial coatings, and environmental remediation. The π - π interactions observed in the C=C bending vibration ($600\text{--}800\text{ cm}^{-1}$) further suggest that these NPs may have enhanced adsorption properties, aligning with previous studies by Zhang et al. (2020).

Overall, the FTIR analysis confirms that bioactive compounds in SBH, including polyphenols, proteins, and sugars, play a significant role in the reduction and stabilisation of Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs. The findings align with existing literature, reinforcing the effectiveness of NP synthesis using SBH while highlighting areas for optimisation.

4.2.2.5 X-Ray Diffraction Spectroscopy

XRD analysis was conducted to determine the crystalline structure of the synthesised Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs. This technique is fundamental in characterising the material properties of NPs, as it provides insights into their crystallinity, phase composition, and structural integrity. The diffraction patterns obtained confirmed the formation of highly crystalline NPs,

with distinct peaks corresponding to specific crystal planes of each material. These findings validate the successful synthesis of the NPs and highlight the influence of synthesis parameters such as temperature, precursor concentration, and reaction time on their crystalline structure (Figure 4.8). Notably, the ability to control these parameters offers opportunities to tailor the crystalline properties of NPs for specific applications, including catalysis, biomedical applications, and electronic devices.



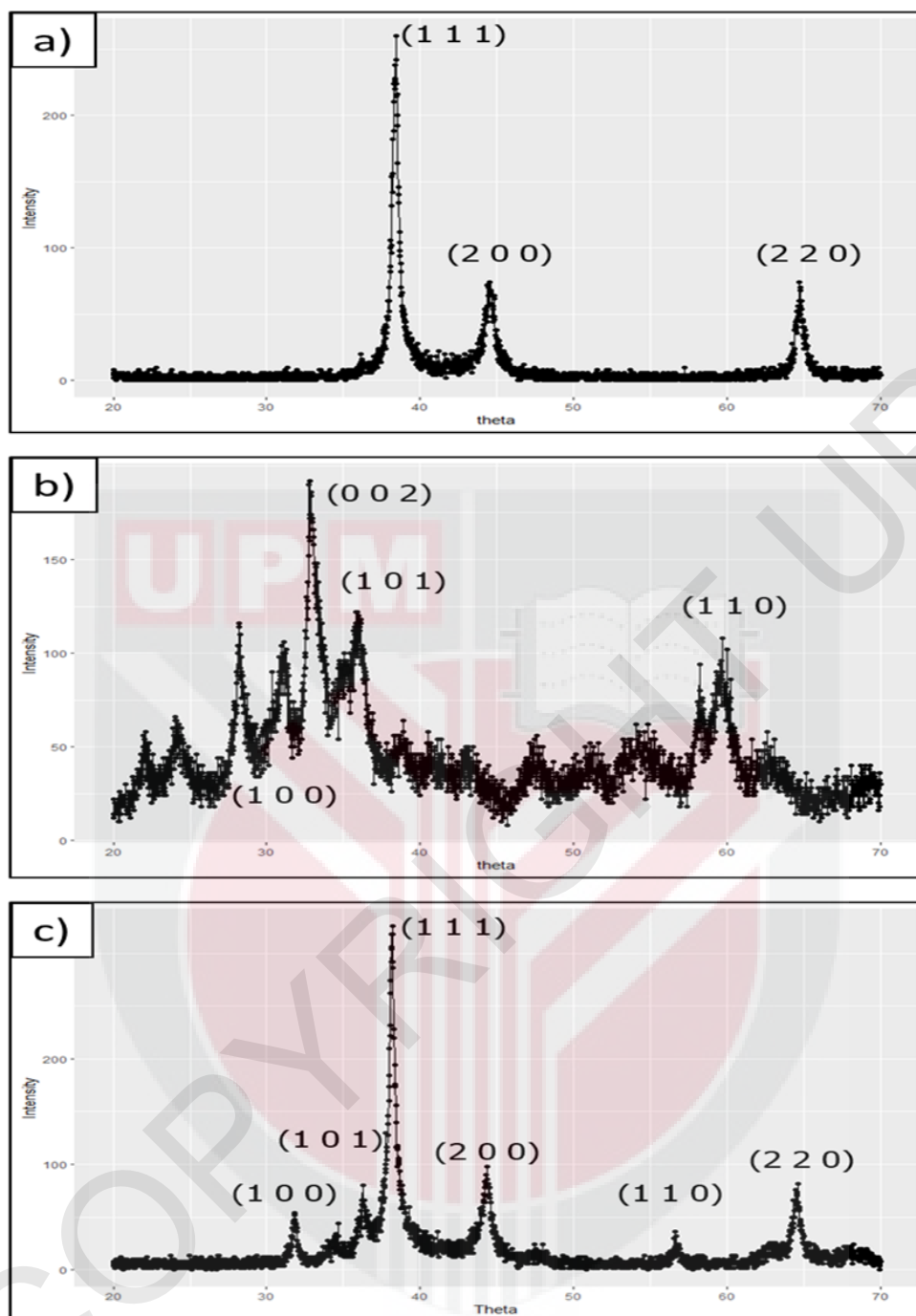


Figure 4.8: XRD patterns and crystallographic analysis of (a) Ag-NPs, (b) ZnO-NPs, and (c) bimetallic Ag/ZnO-NPs.

For the Ag-NPs, three prominent peaks were observed at 2θ values of 38.4° , 44.5° , and 64.7° , corresponding to the (111), (200), and (220) crystallographic planes, respectively. These results closely align with those reported by Sharifi-rad et al. (2020), who identified similar diffraction peaks in green-synthesised

Ag-NPs, confirming the successful reduction of Ag^+ ions. The intense peak at 38.4° suggests a strong (111) orientation, which has been widely associated with enhanced antimicrobial activity due to its higher surface energy and atomic density. Additionally, the stabilisation of Ag-NPs by the bioactive components of SBH is evident, as organic compounds from SBH likely contributed to the formation and structural stability of the NPs. Kyomuhimbo et al. (2019) similarly reported that the organic constituents in natural extracts enhance NPs crystallinity, further supporting the role of SBH as a reducing and stabilising agent. The high signal-to-noise ratio observed in the XRD pattern suggests that the crystalline nature of the synthesised Ag-NPs is well-preserved, with minimal interference from amorphous materials.

In the case of ZnO-NPs, the XRD pattern displayed intense diffraction peaks at 2θ values of 31.1° , 32.8° , 36.0° , and 59.7° , corresponding to the (100), (002), (101), and (110) lattice planes of the hexagonal wurtzite structure. The sharp and well-defined peaks indicate the highly crystalline nature of ZnO-NPs, consistent with the findings of El-Belely et al. (2021). The observed diffraction pattern confirms the purity of the synthesised ZnO-NPs, as no secondary phases or impurity peaks were detected, which aligns with results reported by Afzal et al. (2023). This high degree of crystallinity is crucial for applications in photocatalysis, biosensing, and wound healing, as it enhances the optical and electronic properties of ZnO-NPs. Furthermore, the absence of peak broadening suggests uniform particle size distribution, which is a key factor in maintaining reproducibility in biomedical and industrial applications.

For the bimetallic Ag/ZnO-NPs, the XRD pattern exhibited distinct diffraction peaks at 2θ values of 31.84° , 36.26° , 38.22° , 44.38° , 56.64° , and 64.62° , corresponding to the (100), (101), (111), (200), (110), and (220) crystallographic planes. The presence of peaks associated with both ZnO and Ag phases confirms the successful integration of the two metals into a bimetallic nanocomposite. The coexistence of these crystalline phases without significant peak shifting suggests that the Ag and ZnO structures were preserved during synthesis, ensuring the retention of their unique physicochemical properties. This is consistent with the findings of Afzal et al. (2023), who reported a similar XRD pattern for Ag/ZnO-NPs synthesised via green methods. The formation of a stable bimetallic system is crucial for enhancing antibacterial and photocatalytic efficiency, as the synergistic effects between Ag and ZnO improve charge separation, stability, and overall reactivity.

The XRD results provide strong evidence of the successful synthesis and crystallinity of Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs using SBH. The structural characteristics observed in this study align with prior research on green-synthesised NPs, reaffirming the potential of SBH as a sustainable reducing and stabilising agent. However, despite the overall high crystallinity observed, minor variations in peak intensities suggest potential differences in particle size, morphology, and agglomeration tendencies. The Debye–Scherrer equation was applied to estimate the average crystalline sizes of the synthesised NPs, yielding values of 22.19 nm for Ag-NPs, 21.86 nm for ZnO-NPs, and 24.33 nm for Ag/ZnO-NPs (Table 4.3). These values are within the

expected nanoscale range and are comparable to those reported by previous studies, further validating the effectiveness of the synthesis method.

Table 4.3: The structure and geometric parameters of a) Ag-NPs, b) ZnO-NPs, and c) bimetallic Ag/ZnO-NPs.

a) Ag-NPs					
2θ (°)	θ (°)	FWHM (β), degree	$(\beta, \text{Radian}) = (\beta, \text{Degree}) \times 0.0174$	$\cos \theta$, degree	Crystalline size (nm)
38.42	19.21	0.48	0.008352	0.9443	17.59
44.52	22.26	0.68	0.011832	0.9255	12.67
64.7	32.35	0.26	0.004524	0.8448	36.31
Average					22.19±12.47
b) ZnO-NPs					
2θ (°)	θ (°)	FWHM (β), degree	$(\beta, \text{Radian}) = (\beta, \text{Degree}) \times 0.0174$	$\cos \theta$, degree	Crystalline size (nm)
31.10	15.55	0.58	0.0101	0.9634	14.27
32.78	16.39	0.22	0.0038	0.9594	37.78
35.98	17.99	0.58	0.0101	0.9511	14.46
59.98	29.99	0.44	0.0077	0.8661	20.93
Average					21.86±11.06
c) Bimetallic Ag/ZnO-NPs					
2θ (°)	θ (°)	FWHM (β), degree	$(\beta, \text{Radian}) = (\beta, \text{Degree}) \times 0.0174$	$\cos \theta$, degree	Crystalline size (nm)
31.84	15.92	0.36	0.0063	0.9616	23.04
36.26	18.13	0.62	0.0108	0.9504	13.53
38.22	19.11	0.44	0.0077	0.9449	19.18
44.38	22.19	0.36	0.0063	0.9259	23.93
56.64	28.32	0.26	0.0045	0.8803	34.84
64.62	32.31	0.30	0.0052	0.8452	31.45
Average					24.33±7.82

Note: Results are expressed as the mean $\pm$ Standard Deviation.

The calculated particle sizes indicate that the Ag/ZnO-NPs exhibited slightly larger crystallite sizes compared to the individual Ag-NPs and ZnO-NPs. This could be attributed to the interaction between Ag and ZnO during synthesis, which may have influenced nucleation and growth kinetics. Ismail et al. (2019) suggested that bimetallic NPs tend to exhibit larger crystallite sizes due to enhanced coalescence effects during synthesis. Additionally, while the XRD results confirmed the crystalline nature of the NPs, the presence of a broad

and slightly noisy baseline suggests the influence of amorphous organic compounds from SBH, which may have remained adsorbed onto the NPs' surface. This aligns with findings by Hosny et al. (2017), who reported that plant-based reducing agents often leave residual organic matter on NPs, contributing to peak broadening in XRD analysis.

One potential limitation observed in the bimetallic Ag/ZnO-NPs is the slight overlap of diffraction peaks, which could indicate partial alloying or surface modifications due to the interaction between Ag and ZnO during synthesis. Similar findings were reported by Patil & Chandrasekaran (2020), who suggested that such interactions could alter the electronic and optical properties of the composite material.

4.3 Preparation and Characterisation of Wound Dressings Impregnated with Synthesised Nanoparticles

4.3.1 Wound Dressings Impregnated with Synthesised Nanoparticles

Figure 4.9 shows the wound dressings impregnated with synthesised NPs. The distinct colourations of the gauze dressings correspond directly to the intrinsic optical properties of the NPs, which are influenced by the size, shape, and composition of the NPs, as discussed in Section 4.2.1.

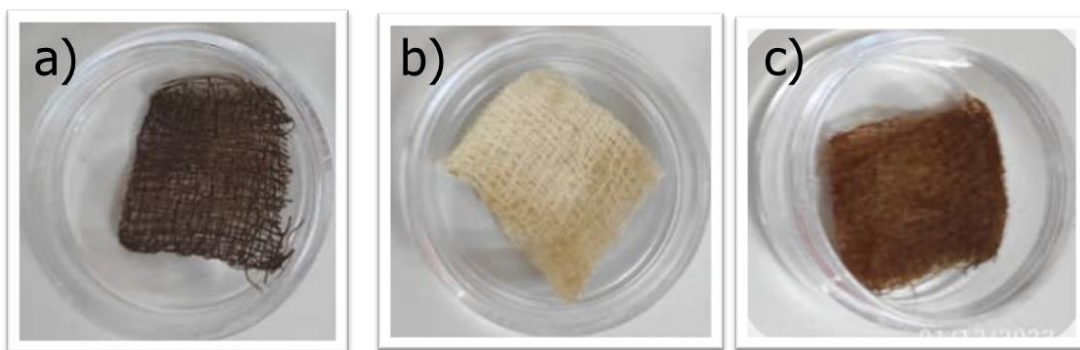


Figure 4.9: Wound Dressing Impregnated with (a) Ag-NPs, (b) ZnO-NPs, and (c) bimetallic Ag/ZnO-NPs.

4.3.2 Characterisation of Wound Dressings Impregnated with Synthesised Nanoparticles

FESEM was employed to analyse the dispersion pattern of the synthesised NPs on the cotton fabrics used in wound dressings. The results revealed that Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs were successfully attached, deposited, and dispersed on the micro-sized threads of the cotton fabrics. However, the distribution was not entirely uniform, with the presence of irregular clump formations, indicating a degree of inhomogeneity in the coating (Fig. 4.10a–c).

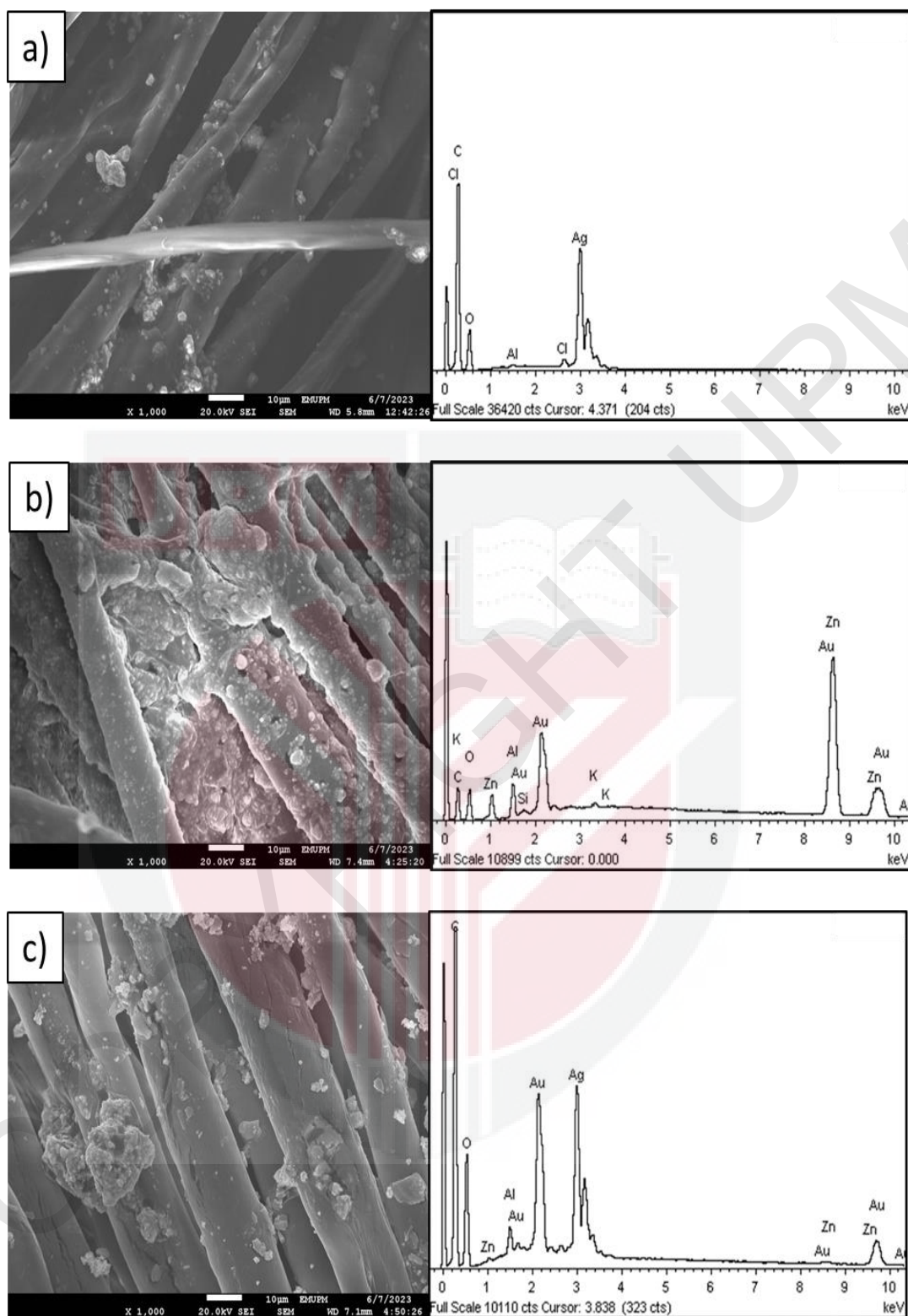


Figure 4.10: FESEM-EDX images of wound dressings impregnated with synthesised NPs; (a) Ag-NPs, (b) ZnO-NPs, and (c) bimetallic Ag/ZnO-NPs at 1000x magnification.

The clustering of NPs can be attributed to their small particle size, which results in high surface energy, prompting aggregation as the particles strive to minimise their total surface area. This tendency aligns with observations reported by Hu et al. (2018), who noted similar irregular agglomerations in ZnO/PVP/PCL and Ag/PVP/PCL nanocomposites. The extent of aggregation may also be influenced by the concentration of NPs and the impregnation method used during the preparation of the wound dressings. Despite the localised clustering, the presence of high-density NP deposits in certain regions could potentially enhance antimicrobial effectiveness due to increased NP availability. However, such non-uniform distribution might also lead to inconsistent antimicrobial performance and reduced sustained release of NPs over time.

EDX confirmed the elemental composition of the NP-impregnated gauzes, providing further validation of the successful integration of synthesised Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs into the cotton fabric. The EDX spectra exhibited characteristic peaks for silver (Ag) and zinc (Zn), corroborating the presence of these elements in their respective NP-treated gauzes. Additionally, peaks corresponding to chlorine (Cl), aluminum (Al), potassium (K), silicon (Si), and oxygen (O) were detected, which can be attributed to the organic and inorganic constituents of SBH used during NP synthesis. This finding is consistent with prior studies, such as those conducted by El-Belely et al. (2021), who reported similar elemental compositions in synthesised NPs as discussed in Section 4.2.2.2. The presence of these elements suggests that SBH components remain adsorbed

on the NP surface, potentially influencing their stability and interaction with biological environments.

Furthermore, gold (Au) peaks appeared in the EDX spectra due to the gold coating applied during FESEM analysis. This coating was necessary to reduce the charging effect typically observed in non-conductive materials like cotton fabric, ensuring enhanced image clarity and resolution. This methodological aspect is standard practice, as reported by Hosny et al. (2017), where gold sputtering was used to improve the visualisation of polymeric nanocomposites.

The FESEM and EDX results substantiate the successful impregnation of Ag-NPs, ZnO-NPs, and Ag/ZnO-NPs onto cotton gauze, demonstrating their potential for wound healing applications. However, the observed NP aggregation presents both advantages and challenges. On one hand, clustered NPs may provide localised antimicrobial hotspots, enhancing bacterial eradication. On the other hand, non-uniform distribution may lead to inconsistent therapeutic effects and hinder the controlled release of NPs, which is crucial for prolonged antimicrobial activity.

Moreover, the presence of residual SBH components in the EDX spectra highlights the potential role of honey-derived bioactive compounds in stabilising the NPs. Prior studies, including Afzal et al. (2023), have shown that polyphenols, flavonoids, and organic acids from SBH can enhance the bioactivity of NPs, contributing to wound healing properties beyond their antimicrobial function. However, further investigations are needed to assess

the long-term stability of these bioorganic coatings and their influence on NP release kinetics.

4.4 Characterisation of Antimicrobial and Cytotoxicity of Synthesised Nanoparticles and Wound Dressings Impregnated with Synthesised Nanoparticles

4.4.1 Antibacterial Assay of Synthesised Nanoparticles and Wound Dressings Impregnated with Synthesised Nanoparticles

S. aureus and *E. coli* were selected for this study because they are the most common pathogens causing soft tissue infections, which can significantly delay wound healing (Govindasamy et al., 2021). *S. aureus* is a leading cause of hospital-acquired infections, while *E. coli* is a primary pathogen associated with burn wound infections (Ullah et al., 2020). Chlorhexidine Hydrochloride was used as the positive control for assessing the antibacterial activity of the synthesised NPs, while commercial Durafiber Ag served as the positive control for evaluating the antibacterial activity of the wound dressings impregnated with the synthesised NPs.

4.4.1.1 Agar Well Diffusion method for Silver, Zinc Oxide, and Bimetallic Silver/Zinc Oxide Nanoparticles

Figures 4.11 and 4.12 illustrate the inhibition zones of the synthesised Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs, alongside chlorhexidine hydrochloride, which served as the positive control. As a widely used antiseptic in clinical settings, chlorhexidine effectively inhibits bacterial growth, providing a reliable benchmark for comparison (Vinay & Chandrasekhar, 2021). The clear

inhibition zones observed confirm the antibacterial efficacy of the synthesised NPs.

According to Table 4.4, chlorhexidine exhibited the largest inhibition zone for *S. aureus* (19.18 ± 0.87 mm), significantly surpassing all tested NPs ($p < 0.05$). Among the NPs, Ag-NPs demonstrated the highest antibacterial activity (16.91 ± 0.40 mm), followed by ZnO-NPs (13.50 ± 0.73 mm) and bimetallic Ag/ZnO-NPs (13.02 ± 0.45 mm). The inhibition zones of ZnO-NPs and bimetallic Ag/ZnO-NPs were not significantly different ($p > 0.05$), suggesting no added benefit from combining Ag and ZnO in a single NP formulation. Similarly, for *E. coli*, the inhibition zones of the positive control (17.41 ± 0.15 mm) and Ag-NPs (17.43 ± 0.26 mm) were statistically comparable ($p > 0.05$), indicating that Ag-NPs exhibit similar antibacterial potency to chlorhexidine. In contrast, ZnO-NPs (14.01 ± 0.95 mm) and bimetallic Ag/ZnO-NPs (14.06 ± 0.54 mm) showed significantly lower activity ($p < 0.05$), with no significant difference between them ($p > 0.05$). These findings highlight Ag-NPs as the most effective antimicrobial agent tested, particularly against *S. aureus*, while their efficacy against *E. coli* matches that of the positive control.

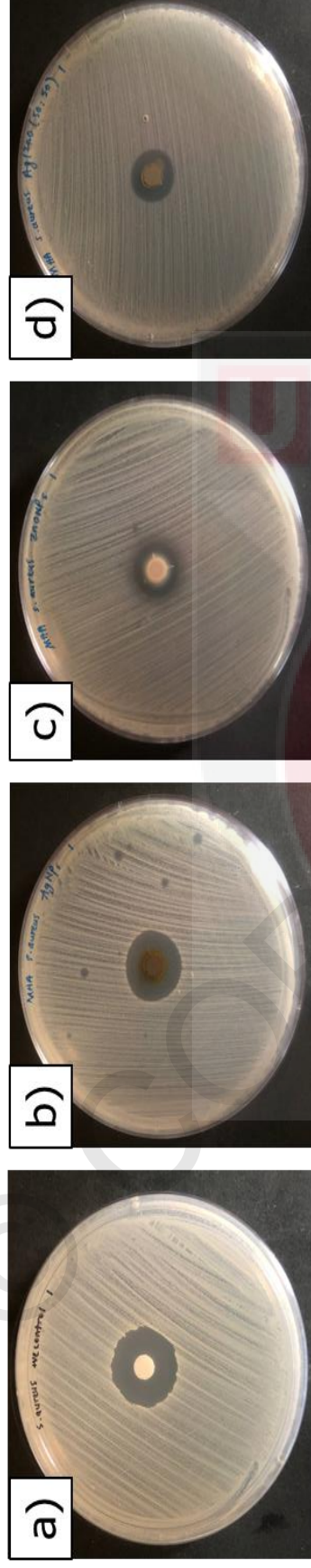


Figure 4.11: Zone of Inhibition (ZOI) of (a) positive control, (b) Ag-NPs, (c) ZnO-NPs, and (d) bimetallic Ag/ZnO-NPs on *S. aureus*.

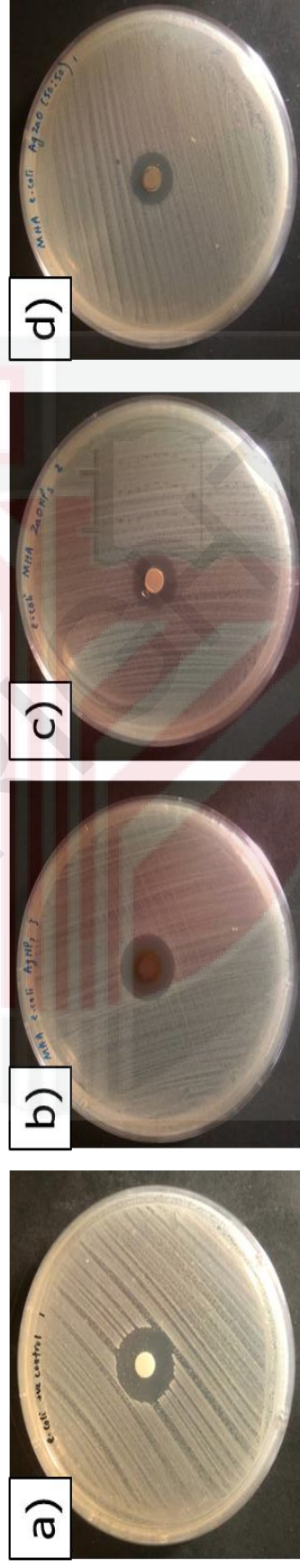


Figure 4.12: Zone of Inhibition (ZOI) of (a) positive control, (b) Ag-NPs, (c) ZnO-NPs, and (d) bimetallic Ag/ZnO-NPs on *E. coli*.

Table 4.4: Zone of inhibition (ZOI) of Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs against Gram-positive and Gram-negative bacteria.

	Bacterial Strains	
	Inhibition zone (mm)	
	<i>S. aureus</i> (ATCC 43300)	<i>E. coli</i> (ATCC 25922)
Positive Control	19.18±0.87 ^a	17.41±0.15 ^a
Ag-NPs	16.91±0.40 ^b	17.43±0.26 ^a
ZnO-NPs	13.50±0.73 ^c	14.01±0.95 ^b
Bimetallic Ag/ZnO-NPs	13.02±0.45 ^c	14.06±0.54 ^b

Note: Results are expressed as the mean of three separate trials ± Standard Deviation.

Similar results were obtained from Jiji et al. (2020), where Ag-NPs has shown higher antibacterial effect in gram positive bacteria, *S. aureus* with comparatively ZOI, 22.33 mm as compared to gram negative bacteria, *E. coli* (12.0 mm).

According to Chugh et al. (2021), the superior antibacterial activity of Ag-NPs can be attributed to their well-documented mechanisms, including membrane disruption, inhibition of protein synthesis, DNA interference, and oxidative stress induction via Ag⁺ ion release. Similarly, ZnO-NPs exert antibacterial effects through ROS generation, which damages bacterial membranes and leads to cell death (Mendes et al., 2022; Spirescu et al., 2021). Additionally, the SBH-based surface coating enhances the antimicrobial properties of NPs by preventing bacterial adhesion and biofilm formation, further strengthening their efficacy (Rajendran & Mani, 2020).

Interestingly, the bimetallic Ag/ZnO-NPs exhibited lower antibacterial activity than their monometallic counterparts, contradicting previous findings by Hu et

al. (2018), which reported enhanced efficacy due to synergistic interactions. This discrepancy may stem from ion interference, where the simultaneous release of Ag^+ and Zn^{2+} reduces their bioavailability and weakens antibacterial effects. Additionally, the distinct antimicrobial mechanisms of Ag-NPs and ZnO-NPs may not complement each other as expected, limiting their combined effectiveness.

Particle size also plays a crucial role in antimicrobial efficacy, as smaller NPs exhibit higher activity due to their increased surface area and greater ability to penetrate bacterial membranes (Yousaf et al., 2020). Thammawithan et al. (2021) and El-Belely et al. (2021) similarly reported that smaller NPs enhance bacterial membrane permeability, as smaller particles have a greater surface area-to-volume ratio than larger particles, leading to more effective interactions and higher antibacterial potency. This aligns with the present study, where monometallic Ag-NPs and ZnO-NPs, being smaller in size, exhibited stronger antibacterial effects compared to bimetallic Ag/ZnO-NPs (Sections 4.2.2.2 & 4.2.2.5).

4.4.1.2 Agar Disc Diffusion Method for Wound Dressings Impregnated with Synthesised Nanoparticles

Similar to the previous section, the antibacterial activity of wound dressings impregnated with synthesised NPs was assessed using the agar disc diffusion method against *S. aureus* and *E. coli*. For comparison, the commercial Durafibre Ag dressing served as the positive control. Durafibre Ag is an absorbent, non-woven dressing containing Ag^+ ions, which are well known for

their antimicrobial properties. Made from cellulose ethyl sulphonate fibres, this dressing maintains a moist wound environment while delivering antimicrobial action. The Ag^+ ions help reduce bacterial bioburden and lower the risk of infection (N.d, 2025). Figures 4.13 and 4.14 illustrate the antibacterial effects, with the corresponding ZOI summarised in Table 4.5.



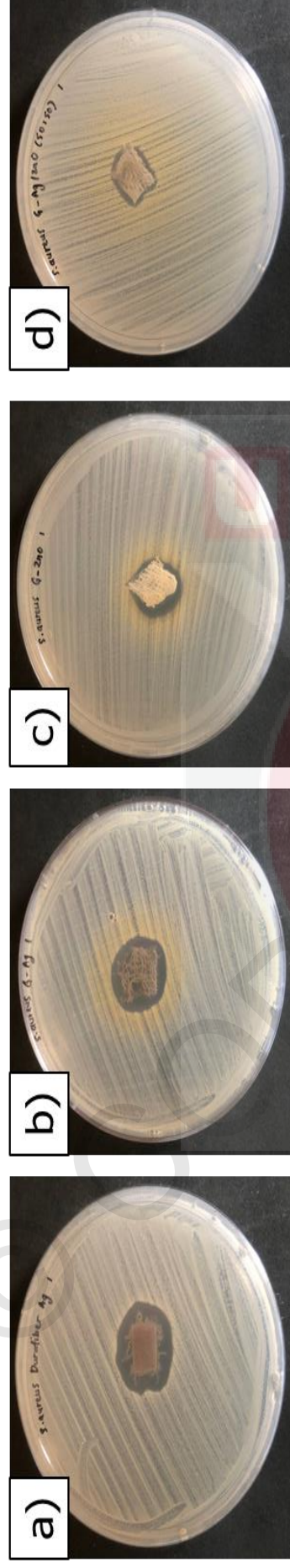


Figure 4.13: Zone of Inhibition (ZOI) of wound dressings (a) positive control, (b) Ag-NPs, (c) ZnO-NPs, and (d) bimetallic Ag/ZnO-NPs on *S. aureus*.

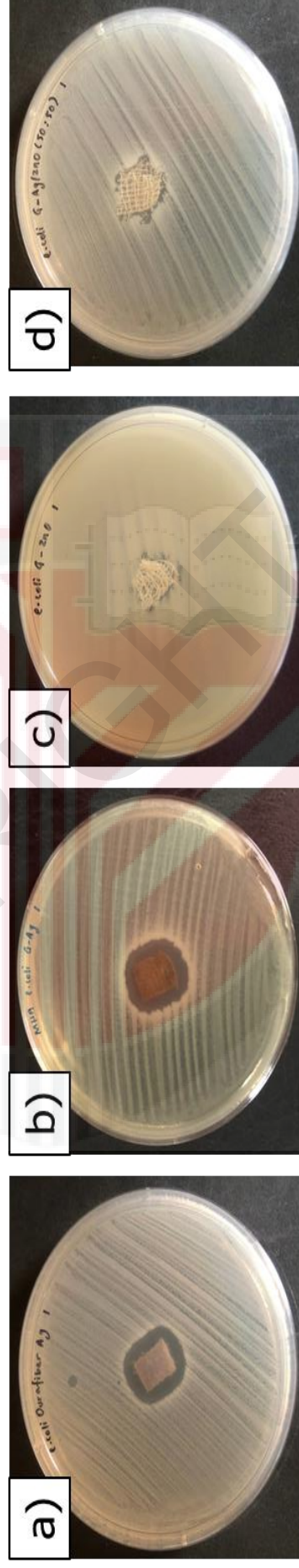


Figure 4.14: Zone of Inhibition (ZOI) of wound dressings (a) positive control, (b) Ag-NPs, (c) ZnO-NPs, and (d) bimetallic Ag/ZnO-NPs on *E. coli*

Table 4.5: Zone of inhibition (ZOI) of wound dressings impregnated with SBH-based NPs (Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs) against Gram-positive and Gram-negative bacteria.

	Bacterial Strains	
	Inhibition zone (mm)	
	<i>S. aureus</i> (ATCC 43300)	<i>E. coli</i> (ATCC 25922)
Positive Control	17.06±0.60 ^a	19.91±1.12 ^a
Ag-NPs	17.56±2.46 ^a	20.19±1.18 ^a
ZnO-NPs	18.41±0.91 ^a	11.55±1.01 ^b
Bimetallic Ag/ZnO-NPs	13.36±0.31 ^b	14.21±1.45 ^b

Note: Results are expressed as the mean of three separate trials ± Standard Deviation.

As presented in Table 4.5, the cotton gauze impregnated with synthesised Ag-NPs exhibited the highest ZOI against *E. coli* (20.19 ± 1.18 mm), a Gram-negative bacterium, while ZnO-NPs demonstrated the most significant antibacterial effect against *S. aureus* (18.41 ± 0.91 mm), a Gram-positive bacterium. These findings suggest that Ag-NPs are particularly effective against *E. coli*, which is known for its antibiotic resistance mechanisms, whereas ZnO-NPs show stronger efficacy against *S. aureus*, highlighting their potential for targeted antibacterial applications.

The statistical analysis further supports these observations. For *S. aureus*, no significant differences ($p > 0.05$) were observed in the inhibition zones of the positive control (17.06 ± 0.60 mm), Ag-NPs (17.56 ± 2.46 mm), and ZnO-NPs (18.41 ± 0.91 mm), indicating comparable antibacterial efficacy. However, the bimetallic Ag/ZnO-NPs dressing exhibited a significantly smaller inhibition zone (13.36 ± 0.31 mm, $p < 0.05$), suggesting reduced effectiveness against *S. aureus*.

Similarly, for *E. coli*, Ag-NPs (20.19 ± 1.18 mm) and the positive control (19.91 ± 1.12 mm) showed no significant difference ($p > 0.05$), indicating similar antibacterial performance. However, ZnO-NPs (11.55 ± 1.01 mm) and bimetallic Ag/ZnO-NPs (14.21 ± 1.45 mm) exhibited significantly lower inhibition zones ($p < 0.05$), demonstrating weaker antibacterial effects. No significant difference was observed between ZnO-NPs and bimetallic Ag/ZnO-NPs ($p > 0.05$), suggesting that combining Ag and ZnO did not enhance antibacterial activity against *E. coli*.

The superior antibacterial performance of Ag-NPs against *E. coli* can be attributed to their ability to release Ag^+ ions, which interact with bacterial cell membranes, causing structural disruption and oxidative stress through the generation of ROS (Chugh et al., 2021). The small size and high surface area-to-volume ratio of Ag-NPs enhance their penetration and interaction with bacterial components, contributing to their potent antimicrobial effects.

In contrast, ZnO-NPs exhibited stronger inhibition against *S. aureus*, likely due to their ROS-generating capability, which induces oxidative stress, damages bacterial membranes, and ultimately leads to cell lysis (Spirese et al., 2021). Although Gram-positive bacteria possess a thick peptidoglycan layer, it remains susceptible to ZnO-NP-induced oxidative damage. Additionally, ZnO-NPs may enhance bacterial uptake, further facilitating their antimicrobial action.

The relatively lower efficacy of ZnO-NPs against *E. coli* can be explained by differences in bacterial cell wall structures. As a Gram-negative bacterium, *E. coli* has an outer membrane that acts as a barrier, limiting NP penetration and resistance to oxidative stress. Conversely, Gram-positive *S. aureus* lacks this outer membrane, making it more vulnerable to ZnO-NP-induced damage (Elhabal et al., 2024). These structural differences play a crucial role in determining bacterial susceptibility to specific NPs.

Variations in ZOI values could also be influenced by the inhomogeneous distribution of NPs on the cotton gauze surface (Section 4.3.2). Uneven dispersion or NPs aggregation may lead to inconsistent antibacterial effects across the dressing, compromising overall efficacy. Additionally, the physical characteristics of the gauze, such as texture and porosity, may affect NPs adherence, potentially diminishing the antimicrobial effect. Achieving a uniform NPs distribution is therefore critical for ensuring consistent antibacterial performance.

4.4.1.2 Minimum Inhibitory Concentration

Based on the antibacterial activity results, the minimum inhibitory concentration (MIC) of synthesised NPs was determined to evaluate the effectiveness of synthesised Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs against *S. aureus* and *E. coli*. The MIC, defined as the lowest concentration of NPs that inhibits bacterial growth, is crucial for understanding the efficacy of these NPs against the tested bacterial strains. A one-way ANOVA followed by

Tukey's post hoc test confirmed significant differences between treatments ($p < 0.05$). Results are expressed as mean $\pm$ standard deviation (SD), with superscript letters denoting statistical significance (Table 4.6).

Table 4.6: MIC of SBH-based NPs.

Bacterial strains	Minimum Inhibition Concentration (MIC), $\mu\text{g/mL}$		
	Ag-NPs	ZnO-NPs	Bimetallic Ag/ZnO-NPs
<i>S. aureus</i> (ATCC 43300)	6.25 $\pm$ 0.00 ^a	1.56 $\pm$ 0.00 ^b	12.50 $\pm$ 0.00 ^c
<i>E. coli</i> (ATCC 25922)	6.25 $\pm$ 0.00 ^a	1.56 $\pm$ 0.00 ^b	6.25 $\pm$ 0.00 ^a

Note: Results are expressed as the mean of three separate trials $\pm$ Standard Deviation.

Among the tested NPs, ZnO-NPs exhibited the lowest MIC values for both *S. aureus* and *E. coli* (1.56 $\pm$ 0.00 $\mu\text{g/mL}$), demonstrating the highest antibacterial potency. This suggests their strong potential for antibacterial applications, particularly against these pathogens. In contrast, bimetallic Ag/ZnO-NPs required a significantly higher MIC for *S. aureus* (12.50 $\pm$ 0.00 $\mu\text{g/mL}$) but had a MIC of 6.25 $\pm$ 0.00 $\mu\text{g/mL}$ for *E. coli*, similar to Ag-NPs (6.25 $\pm$ 0.00 $\mu\text{g/mL}$). These findings indicate that ZnO-NPs are the most effective at lower concentrations, while the bimetallic formulation does not significantly enhance activity against *S. aureus*. This variation may be due to differences in bacterial cell wall structures and resistance mechanisms (Elhabal et al., 2024).

The potent antibacterial effect of ZnO-NPs is likely attributed to their ability to generate ROS, causing bacterial membrane disruption. Ag-NPs, which showed similar MIC values against both bacterial strains, primarily exert their antimicrobial action through the release of Ag⁺ ions, inducing oxidative stress

and membrane damage. Meanwhile, bimetallic Ag/ZnO-NPs provide broad-spectrum antimicrobial activity, particularly effective against Gram-negative bacteria due to the combined action of silver and zinc oxide. This dual mechanism enhances their potential as antimicrobial agents in addressing antibiotic-resistant bacteria.

These results align with existing literature on the antibacterial properties of Ag-NPs and ZnO-NPs, where their effectiveness varies based on bacterial species and NP composition. The high surface area of these NPs enhances bactericidal activity by facilitating greater interaction with bacterial cells (Rajendran & Mani, 2020). Given their strong antimicrobial potential, these nanoparticles hold promise for infection control applications in wound dressings, offering a viable alternative in combating antibiotic-resistant infections.

4.4.1.3 Minimum Bactericidal Concentration

The minimum bactericidal concentration (MBC) was determined by re-inoculating bacterial cultures into fresh LB broth after exposure to the MIC of synthesised Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs. The MBC is the lowest concentration of NPs required to kill 99.9% of the bacterial population. The MBC of the synthesised NPs are presented in Table 4.7 as mean $\pm$ SD, with distinct superscript letters (a, b, and c) denoting statistically significant differences ($p < 0.05$).

Table 4.7: MBC of synthesised NPs.

Bacterial strains	Minimum Bactericidal Concentration (MBC), µg/mL		
	Ag-NPs	ZnO-NPs	Bimetallic Ag/ZnO-NPs
<i>S. aureus</i> (ATCC 43300)	6.25±0.00 ^a	12.50±0.00 ^b	12.50±0.00 ^b
<i>E. coli</i> (ATCC 25922)	12.50±0.00 ^a	3.12±0.00 ^b	6.25±0.00 ^c

Note: Results are expressed as the mean of three separate trials ± Standard Deviation.

For *S. aureus* (ATCC 43300), the study revealed that Ag-NPs exhibited the lowest MBC at 6.25 ± 0.00 µg/mL. This value was significantly lower than that of ZnO-NPs and bimetallic Ag/ZnO-NPs, both of which demonstrated MBC values of 12.50 ± 0.00 µg/mL ($p < 0.05$). Notably, the MBC values for Ag-NPs and bimetallic Ag/ZnO-NPs were identical to their respective MIC, indicating that these NPs not only effectively inhibit bacterial growth but also achieve complete bactericidal effects at these specific concentrations. In contrast, ZnO-NPs required a higher concentration to exhibit bactericidal activity compared to their ability to inhibit bacterial growth, as evidenced by the observed disparity between the MIC and MBC values. This difference suggests a more complex mechanism of action for ZnO-NPs, highlighting the potential for varying interactions with the bacterial cells.

For the strain of *E. coli* (ATCC 25922), the evaluation of the MBC revealed that ZnO-NPs exhibited the lowest MBC value of 3.12 ± 0.00 µg/mL. This finding was statistically significant when compared to the MBC of Ag-NPs, which was determined to be 12.50 ± 0.00 µg/mL, as well as the bimetallic Ag/ZnO-NPs, which showed an MBC of 6.25 ± 0.00 µg/mL ($p < 0.05$). Notably, the MBC values for both Ag-NPs and ZnO-NPs were approximately 50% higher than

their respective MICs. This indicates a marked difference between the concentrations required to inhibit bacterial growth and those needed to achieve bactericidal effects. In contrast, the MBC of the bimetallic Ag/ZnO-NPs was found to be equal to its MIC. This result underscores the effectiveness of the bimetallic formulation, as it demonstrates potent bactericidal activity at the same concentration that is sufficient for inhibiting bacterial growth.

The relationship between the MIC and the MBC is critical in evaluating the overall antibacterial efficacy of various agents. A close MIC-MBC ratio signifies that the agent can effectively eradicate bacteria at relatively low concentrations, which is particularly advantageous in clinical settings. For instance, this is evident in the behavior of Ag-NPs and bimetallic Ag/ZnO-NPs, where their ability to maintain a narrow MIC-MBC gap suggests efficient bacterial eradication with minimal dosage. In contrast, the significant MIC-MBC gap observed for ZnO-NPs indicates that although these NPs can successfully inhibit bacterial growth, achieving complete bactericidal action requires a much higher concentration. This necessary increase in dosage may lead to clinical concerns, such as the potential for developing bacterial resistance over time, as higher concentrations of antimicrobial agents can create selective pressure on bacterial populations (Zare et al., 2017).

The enhanced antibacterial performance of bimetallic Ag/ZnO-NPs can be attributed to their synergistic mechanism, which involves the intricate interplay between the two metals. The combination of silver and zinc oxide not only improves charge separation but also reduces bandgap energy, which plays a

crucial role in minimising electron-hole recombination. This process, in turn, significantly enhances the antimicrobial effects of the NPs (Rajendran & Mani, 2020). The silver ions released from Ag-NPs are known for their broad-spectrum antibacterial activity, effectively targeting various pathogenic bacteria. Simultaneously, zinc oxide generates hydrogen peroxide, a potent bactericidal agent that further contributes to the overall antimicrobial action. This dual mechanism of action, where both silver and zinc oxide work in tandem, significantly boosts the bactericidal efficacy of bimetallic Ag/ZnO-NPs. As a result, these NPs hold great potential for applications in medical settings, particularly in wound dressings and coatings for medical devices, where preventing bacterial infection is critical. Their enhanced antibacterial properties make them an attractive choice for developing more effective antimicrobial agents.

From a wound care perspective, these findings suggest that synthesised Ag-NPs and bimetallic Ag/ZnO-NPs are particularly effective at relatively low concentrations, making them promising candidates for infection control and wound healing applications. The ability to achieve significant antibacterial activity at such low concentrations is crucial, as it minimises the risk of cytotoxicity to surrounding healthy tissues while maximising therapeutic effects. However, the higher MBC values for ZnO-NPs highlight the critical need for precise concentration optimisation to ensure effective bactericidal action. This is essential to strike a balance between efficacy and the potential risk of developing bacterial resistance, which is an increasing concern in modern medicine. The synergistic properties of bimetallic Ag/ZnO-NPs offer

not only a promising alternative for innovative wound dressings but also enhanced antibacterial performance, making them particularly suitable for use in clinical applications. Moreover, their unique combination of properties may lead to improved patient outcomes, reduced healing times, and a decrease in the incidence of wound infections, which are common complications in both acute and chronic wounds.

Remarkably, the green synthesised Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs using SBH showed significant bacteriolytic activity compared with several other NPs synthesised using others green sources. Table 4.8 shows the comparative analysis of current and previous studies on the antibacterial activity on selected pathogens.

Table 4.8: Comparative analysis of current and previous studies on the antibacterial activity.

	Current study		Bandeira et al. (2021)	Li et al. (2021)	Jha et al. (2023)	Jomehzadeh et al. (2021)	Shamshad et al. (2020)
Green sources	Stingless honey	bee	<i>Ilex paraguariensis</i> leaves extract	Sericin	<i>Camellia sinensis</i> leaf extract	<i>Pistacia atlantica</i> resin extract	<i>Ficus palmata</i>
NPs used	1) Ag-NPs 2) ZnO-NPs 3) bimetallic Ag/ZnO-NPs.	ZnO-NPs	1) Ag-NPs 2) ZnO-NPs 3) bimetallic Ag/ZnO-NPs.	1) ZnO-NPs 2) Bimetallic Ag/ZnO-NPs	1) Ag-NPs 2) ZnO-NPs 3) bimetallic Ag/ZnO-NPs.	1) Ag-NPs 2) ZnO-NPs	1) Ag-NPs 2) ZnO-NPs
Pathogens used	1) <i>S. aureus</i> 2) <i>E. coli</i>	1) <i>S. aureus</i> 2) <i>E. coli</i>	1) <i>S. aureus</i> 2) <i>E. coli</i>	1) <i>S. aureus</i> 2) <i>E. coli</i>	1) <i>S. aureus</i> 2) <i>E. coli</i> 3) <i>Klebsiella pneumoniae</i> 4) <i>Proteus mirabilis</i>	1) <i>S. aureus</i> 2) <i>E. coli</i> 3) <i>Klebsiella pneumoniae</i> 4) <i>Bacillus subtilis</i> 5) <i>Pseudomonas aeruginosa</i> 6) Resistant <i>Pseudomonas aeruginosa</i> 7) Resistant <i>E. coli</i> 8) Resistant <i>Streptococcus haemolyticus</i>	1) <i>S. aureus</i> 2) <i>E. coli</i> 3) <i>Klebsiella pneumoniae</i> 4) <i>Bacillus subtilis</i> 5) <i>Pseudomonas aeruginosa</i> 6) Resistant <i>Pseudomonas aeruginosa</i> 7) Resistant <i>E. coli</i> 8) Resistant <i>Streptococcus haemolyticus</i>

Table 4.8: Continued

	Current study	Bandeira et al. (2021)	Li et al. (2021)	Jha et al. (2023)	Jomehzadeh et al. (2021)	Shamshad et al. (2020)
ZOI, mm	<i>S. aureus</i>: 1) Ag-NPs – 16.91; 17.56 2) ZnO-NPs – 13.5; 18.41 3) bimetallic Ag/ZnO-NPs – 13.02; 13.36 <i>E. coli</i>: 1) Ag-NPs – 17.43; 20.19 2) ZnO-NPs – 14.01; 11.55 3) bimetallic Ag/ZnO-NPs – 14.06; 14.21	Nm	<i>S. aureus</i>: 1) Ag-NPs – 3.4 2) ZnO-NPs – 0.4 3) bimetallic Ag/ZnO-NPs – 4.5 <i>E. coli</i>: 1) Ag-NPs – 9.3 2) ZnO-NPs – 2.5 3) bimetallic Ag/ZnO-NPs – 10.5	<i>S. aureus</i>: 1) ZnO-NPs – 12.0 2) bimetallic Ag/ZnO-NPs – 14.0 <i>E. coli</i>: 1) ZnO-NPs – - 2) bimetallic Ag/ZnO-NPs – 13.0	<i>S. aureus</i>: 1) Ag-NPs – 13.0 2) ZnO-NPs – 14.0 3) bimetallic Ag/ZnO-NPs – 16.0 <i>E. coli</i>: 1) Ag-NPs – 12.5 2) ZnO-NPs – 17.0 3) bimetallic Ag/ZnO-NPs – 18.6 <i>Klebsiella pneumoniae</i>: 1) Ag-NPs – 9.4 2) ZnO-NPs – 13.6 3) bimetallic Ag/ZnO-NPs – 15.0	

Table 4.8: Continued

	Current study	Bandeira et al. (2021)	Li et al. (2021)	Jha et al. (2023)	Jomehzadeh et al. (2021)	Shamshad et al. (2020)
					<i>Proteus mirabilis</i> : 1) Ag-NPs – 15.0 2) ZnO-NPs – 17.5 3) bimetallic Ag/ZnO-NPs – 21.0	
MIC, $\mu\text{g/mL}$	<i>S. aureus</i>: 1) Ag-NPs – 6.25 2) ZnO-NPs – 1.56 3) bimetallic Ag/ZnO-NPs – 12.50 <i>E. coli</i>: 1) Ag-NPs – 6.25 2) ZnO-NPs – 1.56 3) bimetallic Ag/ZnO-NPs – 6.25	<i>S. aureus</i>: 35 <i>E. coli</i>: 100	Nm	Nm	Nm	<i>S. aureus</i>: 1) Ag-NPs – 11.1 2) ZnO-NPs – 100.0 <i>E. coli</i>: 1) Ag-NPs – 11.1 2) ZnO-NPs – - <i>Klebsiella pneumoniae</i>: 1) Ag-NPs – - 2) ZnO-NPs – - <i>Bacillus subtilis</i>: 1) Ag-NPs – 100 2) ZnO-NPs – - <i>Pseudomonas aeruginosa</i>: 1) Ag-NPs – 100 2) ZnO-NPs – -

Table 4.8: Continued

	Current study	Bandeira et al. (2021)	Li et al. (2021)	Jha et al. (2023)	Jomehzadeh et al. (2021)	Shamshad et al. (2020)
						Resistant <i>Pseudomonas aeruginosa</i> : 1) Ag-NPs – 3.7 2) ZnO-NPs – 11.1 Resistant <i>E. coli</i> : 1) Ag-NPs – 100 2) ZnO-NPs – 3.7 Resistant <i>Streptococcus haemolyticus</i> 1) Ag-NPs – 3.7 ZnO-NPs – 11.1
MBC, $\mu\text{g/mL}$	<i>S. aureus</i> : 1) Ag-NPs – 6.25 2) ZnO-NPs – 12.5 3) bimetallic Ag/ZnO-NPs – 12.5 <i>E. coli</i> : 1) Ag-NPs – 12.5 2) ZnO-NPs – 3.12 3) bimetallic Ag/ZnO-NPs – 6.25	Nm	Nm	Nm	Nm	Nm

Nm – not mention.

The antimicrobial efficacy of SBH synthesised NPs in the current study demonstrated superior performance compared to previous research. The Ag-NPs exhibited larger inhibition zones against *S. aureus* (16.91–17.56 mm) and *E. coli* (17.43–20.19 mm) compared to those reported by Li et al. (2021) (3.4 mm and 9.3 mm, respectively) and Jomehzadeh et al. (2021) (13.0 mm and 12.5 mm, respectively). This enhanced antibacterial activity suggests that the bioactive compounds in SBH played a significant role in stabilising and enhancing the efficacy of Ag-NPs, potentially through synergistic antimicrobial mechanisms. Similarly, ZnO-NPs in the current study demonstrated superior performance against *S. aureus* (13.5–18.41 mm) compared to previous reports, particularly Li et al. (2021), who observed minimal inhibition (0.4 mm). This finding indicates that the synthesis method and bioactive components in SBH significantly influenced ZnO-NP effectiveness, possibly by improving their stability, dispersion, and bioavailability.

However, while the bimetallic Ag/ZnO-NPs in Jomehzadeh et al. (2021) exhibited greater efficacy against *E. coli* (18.6 mm) than in the current study (14.06–14.21 mm), the latter's ZnO-NPs displayed the lowest MIC against *S. aureus* (1.56 µg/mL) among all studies, highlighting its superior antibacterial potency even at lower concentrations. Similarly, the ZnO-NPs from this study demonstrated exceptional efficacy against *E. coli* (MIC 1.56 µg/mL), outperforming all compared studies. Additionally, the Ag-NPs in the current study (MIC 6.25 µg/mL for both pathogens) showed better antimicrobial activity than those reported by Shamshad et al. (2020) (11.1 µg/mL), further

underscoring the influence of synthesis conditions and stabilizing agents on NP performance.

Interestingly, unlike some studies where bimetallic Ag/ZnO-NPs consistently outperformed mono-metallic NPs, the current study revealed that individual NPs, particularly ZnO-NPs, sometimes exhibited superior antibacterial activity. This deviation from expected trends suggests that factors such as NP size, morphology, surface charge, and the interaction between Ag and ZnO within the bimetallic system may influence their antimicrobial mechanisms as being discussed in previous section. While bimetallic NPs are often assumed to benefit from combined antimicrobial effects, their efficacy can depend on the synthesis approach and the ratio of metal components.

4.4.2 *In-vitro* Cytotoxicity

The cytotoxicity of synthesised Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs was evaluated using the MTT assay in HaCaT (human keratinocytes), 3T3 (mouse fibroblasts), and MRC5 (human lung fibroblasts) cells after 72 hours of exposure. Figure 4.15 (a–c) illustrates the concentration-dependent reduction in cell viability across these cell lines. Statistical analysis (ANOVA followed by Tukey's HSD test) revealed significant differences in cytotoxic effects among the NPs.

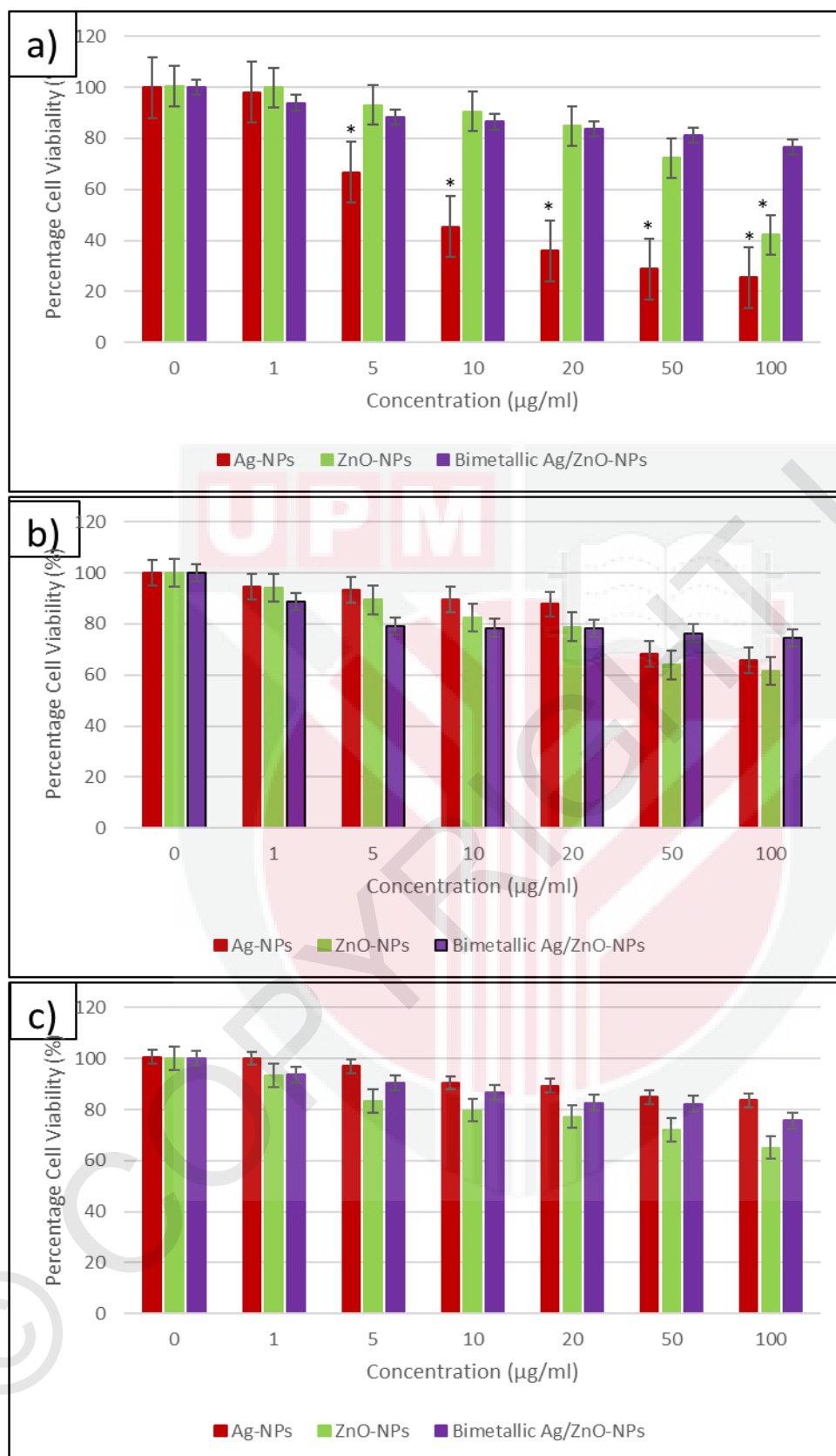


Figure 4.15: *In-vitro* Cytotoxicity of Wound Dressings Impregnated with SBH-based NPs on (a) HaCaT, (b) 3T3, and (c) MRC5 cells after 72 h of exposure. The data are presented as the mean $\pm$ SE and significance indicated by (*) at $p < 0.05$.

In HaCaT cells (Figure 4.15 (a)), Ag-NPs exhibited a strong dose-dependent cytotoxic effect, significantly reducing cell viability as the concentration increased ($p < 0.05$). ZnO-NPs and bimetallic Ag/ZnO-NPs showed moderate toxicity, with cell viability remaining above 80% at lower concentrations. However, at higher concentrations (50 and 100 $\mu\text{g/mL}$), ZnO-NPs led to a substantial reduction in viability compared to the control ($p < 0.01$). The IC_{50} values for Ag-NPs and ZnO-NPs were determined as 10 $\mu\text{g/mL}$ and 85 $\mu\text{g/mL}$, respectively. These findings align with Hu et al. (2018), who reported that smaller NPs generally exhibit greater cytotoxicity due to increased cellular uptake and ROS generation.

For 3T3 cells (Figure 4.15 (b)), viability remained above 80% across all treatments, with no statistically significant differences at lower concentrations ($p > 0.05$). At higher concentrations (50 and 100 $\mu\text{g/mL}$), ZnO-NPs and bimetallic Ag/ZnO-NPs induced a mild reduction in viability, though less pronounced than in HaCaT cells. These results indicate that 3T3 cells exhibited greater tolerance to NPs exposure.

In MRC5 cells (Figure 4.15 (c)), a similar trend was observed. Ag-NPs slightly reduced viability at higher concentrations, while ZnO-NPs caused a significant decline at 50 and 100 $\mu\text{g/mL}$ ($p < 0.05$). Bimetallic Ag/ZnO-NPs maintained relatively stable viability across all tested concentrations, suggesting minimal cytotoxic effects.

The cytotoxicity of Ag-NPs and ZnO-NPs in HaCaT cells was primarily attributed to ROS generation. ZnO-NPs catalysed excessive ROS production, leading to oxidative stress, lipid peroxidation, protein denaturation, and DNA damage, ultimately resulting in cell necrosis. Meanwhile, Ag-NPs triggered apoptosis via the mitochondrial pathway, involving cytochrome c release and JNK activation. These findings are consistent with studies by Rajendran & Mani (2020), who linked cellular damage to elevated ROS levels and mitochondrial dysfunction.

Despite their superior antimicrobial properties, monometallic Ag-NPs and ZnO-NPs induced significant cytotoxicity compared to bimetallic Ag/ZnO-NPs. Notably, bimetallic Ag/ZnO-NPs exhibited no cytotoxic effects in any cell line at concentrations up to 100 $\mu\text{g/mL}$, demonstrating superior biocompatibility. This enhanced biocompatibility may be attributed to the controlled release of metal ions, reducing oxidative stress while maintaining antimicrobial efficacy, as suggested by Elhabal et al. (2024).

These findings underscore the importance of balancing antimicrobial effectiveness and cytocompatibility when developing NPs-based wound dressings. While Ag-NPs and ZnO-NPs provide strong antibacterial activity, their higher cytotoxicity towards HaCaT cells suggests potential limitations in wound healing applications. Conversely, bimetallic Ag/ZnO-NPs present a promising alternative, offering a balance between antimicrobial potency and biocompatibility, making them a safer and more effective candidate for wound dressings.

4.5 Evaluation of *In-vivo* Wound Healing Activity

4.5.1 Wound Closure

Figure 4.16 illustrates the progression of wound healing in Sprague-Dawley rats treated with synthesised NPs-impregnated wound dressings. Wounds were cleaned daily with sterile saline, and wound contraction was measured at days 4, 8, 12, 16, and 20.

During the initial phase of wound healing, untreated wounds (negative control) and those treated with commercial Durafibre Ag (positive control) exhibited severe inflammation, characterised by bleeding and purulence. By day 8, wound treated with cotton gauze impregnated with synthesised bimetallic Ag/ZnO-NPs showed accelerated wound contraction, likely due to reduced capillary permeability and enhanced wound bed dehydration. This finding supports the results of Kantipudi et al. (2018) and Majhi et al. (2020), who reported that bimetallic Ag/ZnO has prominent role in the earlier stages of wound healing process.

According to Majhi et al. (2020), Ag/ZnO-NPs enhanced HaCaT keratinocyte proliferation and migration, leading to faster in vitro wound closure. However, the study also highlighted the need for concentration optimisation, as higher Ag/ZnO concentrations could be detrimental. By day 16, wounds treated with synthesised NPs dressings appeared clean and free from debris, indicating effective exudate absorption. This observation aligns with the findings of Khan

et al. (2021) and Burduşel et al. (2018), which demonstrated the effectiveness of silver-based wound dressings in managing exudate.



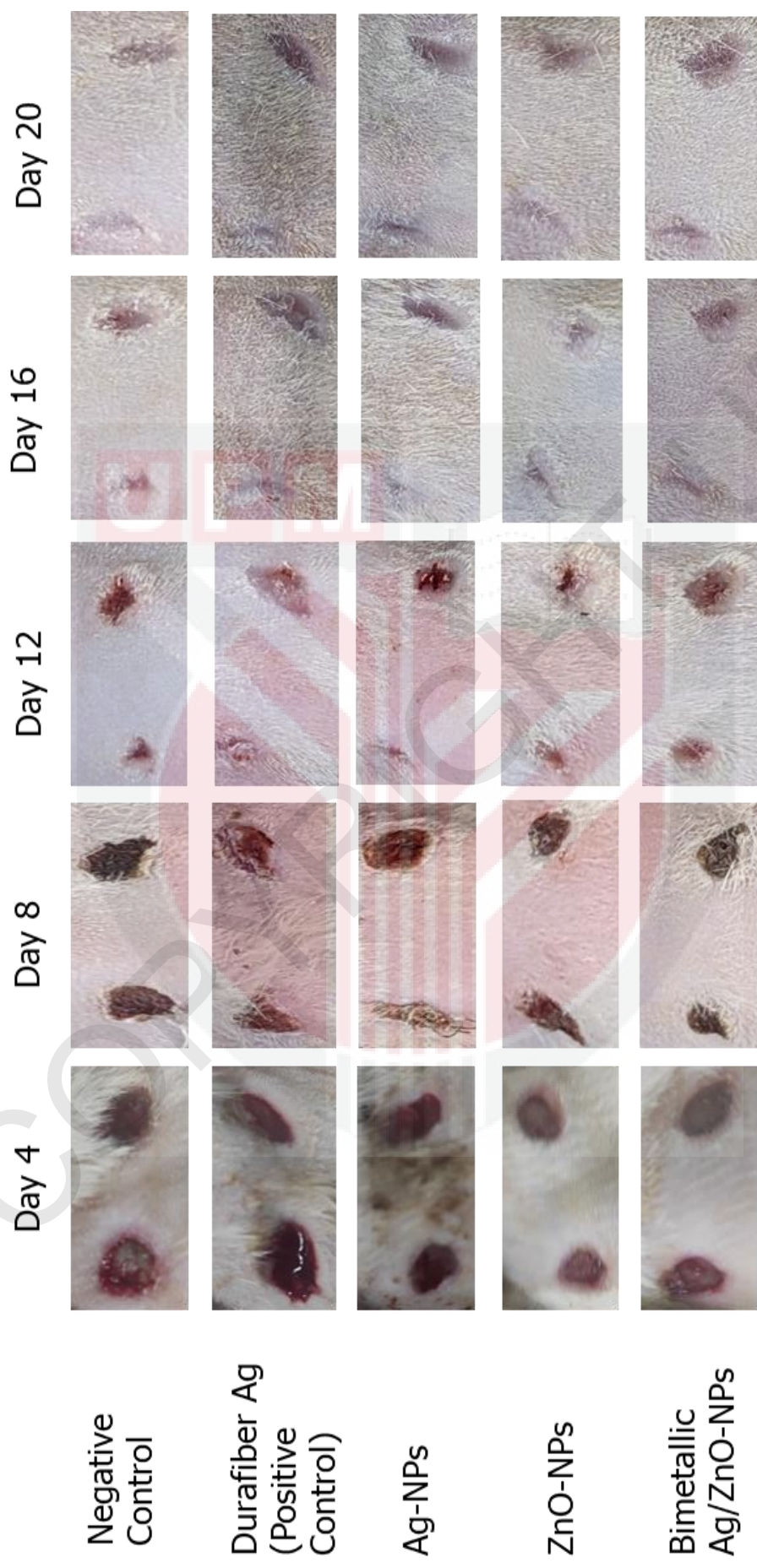


Figure 4.16: Progress of wound healing in negative-control (distilled water), positive control (Durafibre Ag), and synthesised NPs (Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs) at days 4, 8, 12, 16, and 20 post wounding.

For further clarification, figure 4.17 and Table 4.9 illustrate these wound closure trends, showing significant healing progression from Day 4 onward and complete epithelialisation by Day 12.

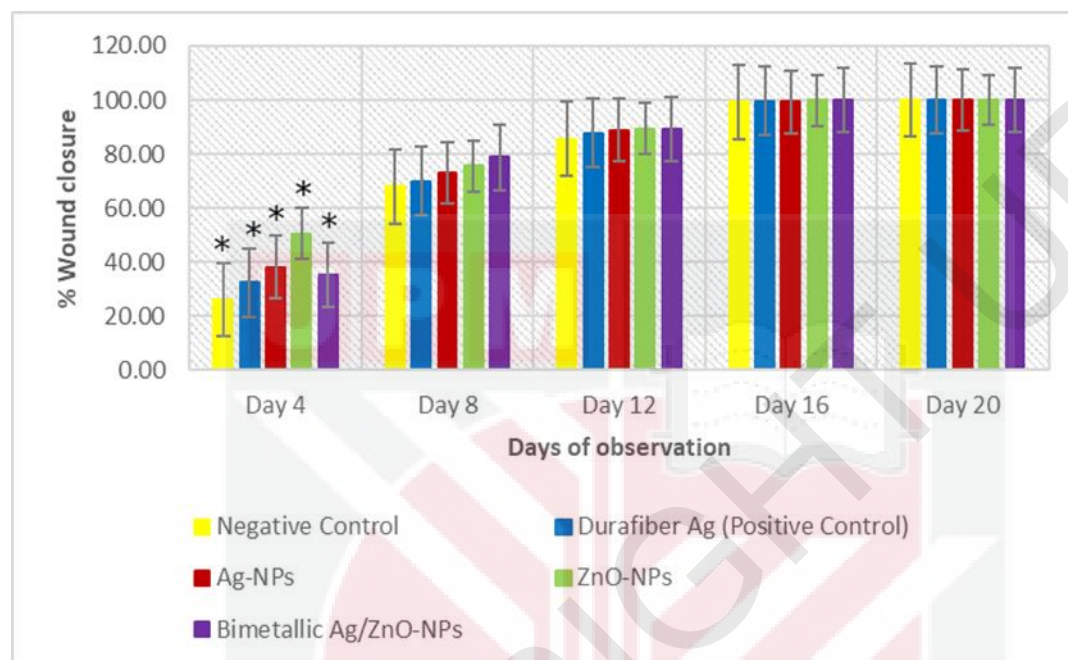


Figure 4.17: Effect on percentage wound closure in negative-control (distilled water), positive control (Durafibre Ag), and synthesised NPs (Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs) at day 4, 8, 12, 16, and 20 post wounding. The data are presented as the mean $\pm$ SE and significance indicated by (*) at $p < 0.05$.

The statistical analysis highlights significant differences among the treatment groups. At Day 0, all groups had an equal initial wound size of approximately 50.24 mm², with no statistically significant differences ($p > 0.05$), ensuring a uniform baseline for comparison. By Day 4, distinct differences in wound healing emerged. The ZnO-NPs treatment group exhibited the highest wound closure (50.54%), followed by Ag-NPs (38.08%), bimetallic Ag/ZnO-NPs (35.13%), and Durafiber Ag (32.21%). In contrast, the negative control group showed the slowest healing progress (26.05% wound closure).

Statistical analysis ($p < 0.05$) confirmed that ZnO-NPs significantly enhanced early-stage wound healing compared to other treatments. This accelerated healing effect may be attributed to the antimicrobial and anti-inflammatory properties of ZnO-NPs, which facilitate ROS formation, enhance cell adhesion, and promote migration through growth factor-mediated pathways (Elhabal et al., 2024). ZnO-NPs are also known to regulate autolytic debridement, keratinocyte migration, and oxygen transport, which contribute to faster wound drying and closure (Norouzi et al., 2021). This finding aligns with the study by Abdelghafar et al. (2022), which reported that wounds treated with ZnO-NPs exhibited the fastest healing rate in the early stage, achieving 58.3% closure by day 6 and complete wound closure by day 14.

Table 4.9. Evaluation of the wound area mm² of different groups over a period of 20 days.

Day	Wound size (mm ²)				
	Negative Control	Durafibre Ag (Positive Control)	Ag-NPs	ZnO-NPs	Bimetallic Ag/ZnO-NPs
0	50.24±0.00 ^a	50.24±0.01 ^a	50.24±0.02 ^a	50.24±0.03 ^a	50.24±0.04 ^a
4	37.15±7.24 ^a	34.06±10.25 ^b	31.09±13.41 ^b	24.85±11.14 ^c	32.59±14.21 ^b
8	16.11±6.32 ^a	15.84±8.05 ^a	13.54±8.47 ^b	12.33±6.11 ^b	10.71±6.36 ^c
12	7.23±4.20 ^a	6.13±3.97 ^a	5.61±3.61 ^a	5.33±3.54 ^a	5.40±3.69 ^a
16	0.38±0.34 ^a	0.18±0.06 ^a	0.36±0.24 ^a	0.09±0.03 ^a	0.00±0.00 ^a
20	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a

Note: Results are expressed as the mean of three separate trials ± Standard Deviation.

The findings for SBH-synthesised Ag-NPs are consistent with those reported by Deeba et al. (2023). Their study demonstrated that wounds treated with Ag-NPs synthesised using *Lepidium didymium* showed a statistically significant

wound contraction of 36.14% by day 3. Similarly, in the current study, the percentage of wound contraction reached 38.08% by day 4. This suggests that despite differences in the materials used for synthesis, the efficacy of the synthesised NPs remains comparable.

By Day 8, all groups exhibited substantial wound healing. Synthesised bimetallic Ag/ZnO-NPs (78.68% closure) and ZnO-NPs (75.46% closure) showed superior healing compared to Ag-NPs, positive control, and the negative control. The enhanced wound closure observed with bimetallic Ag/ZnO-NPs suggests a synergistic effect between Ag-NPs and ZnO-NPs, improving antimicrobial activity and tissue regeneration. Statistical analysis ($p < 0.05$) confirmed significant differences among the treatment groups, with synthesised NPs demonstrating faster wound contraction.

By Day 12, the wound sizes among the treated groups were minimal, with ZnO-NPs (5.33 mm²), bimetallic Ag/ZnO-NPs (5.40 mm²), and Ag-NPs (5.61 mm²) showing no statistically significant differences ($p > 0.05$). This suggests that while ZnO-NPs and bimetallic Ag/ZnO-NPs facilitated faster healing in earlier stages, all NPs-based treatments were equally effective by this phase.

By Day 16, near-complete healing was observed in all groups. The bimetallic Ag/ZnO-NPs group achieved full wound closure (0.00 mm²), while ZnO-NPs (0.09 mm²), and Durafiber Ag (0.18 mm²) showed minimal residual wounds. Ag-NPs and the negative control exhibited minor remaining wounds, but no statistically significant differences were observed at this stage ($p > 0.05$). By

Day 20, all treatment groups achieved 100% wound closure, confirming the effectiveness of the treatments

4.5.2 Histopathological Analysis

Histopathological analysis was conducted on skin samples from various groups on the 20th day to assess the impact of synthesised NPs on wound healing. Hematoxylin and eosin (H&E) staining was used to evaluate the formation of granulation tissue and re-epithelialisation. Figure 4.18 shows that by day 20, skin samples from both treated and untreated rats exhibited normal histological features. The wounds displayed a distinct layer of orthokeratotic keratinocytes in the stratum corneum, a well-organised dermis, and intact adnexal structures, with no signs of inflammation such as edema or cellular infiltration. Complete wound closure was characterised by the absence of visible epithelial defects or adherent scabs.

The epidermis showed noticeable thickening, a result of increased basal cell mitotic activity, while the dermis appeared denser, likely due to enhanced fibroblast activity. The presence of a thicker epidermis and a denser dermis indicates an accelerated healing process facilitated by synthesised NPs. Takzaree et al. (2016) highlighted the role of fibroblasts in collagen production, which is crucial for wound contraction, while Abbas et al. (2023) emphasised the importance of collagen content in tissue proliferation and wound healing. Consistent with these findings, the observed denser dermis suggests

enhanced collagen deposition and accelerated wound healing due to the synthesised NPs.

Furthermore, the histological examination revealed a more mature arrangement of collagen fibres, which likely contributed to the accelerated healing process. The tissues exhibited a well-developed epidermis consisting of two to three cell layers and a robust dermis, with typical structures such as hair follicles (HF), sebaceous glands (SG), and blood vessels (BV) evident. This finding supports previous studies that underscored the critical role of angiogenesis in the regeneration of both the epidermis and dermis, including the development of hair follicles (El-Aassar et al., 2020). The mature collagen fibre arrangement and well-organised skin structures observed in this study underscore the effective wound healing promoted by synthesised NPs.

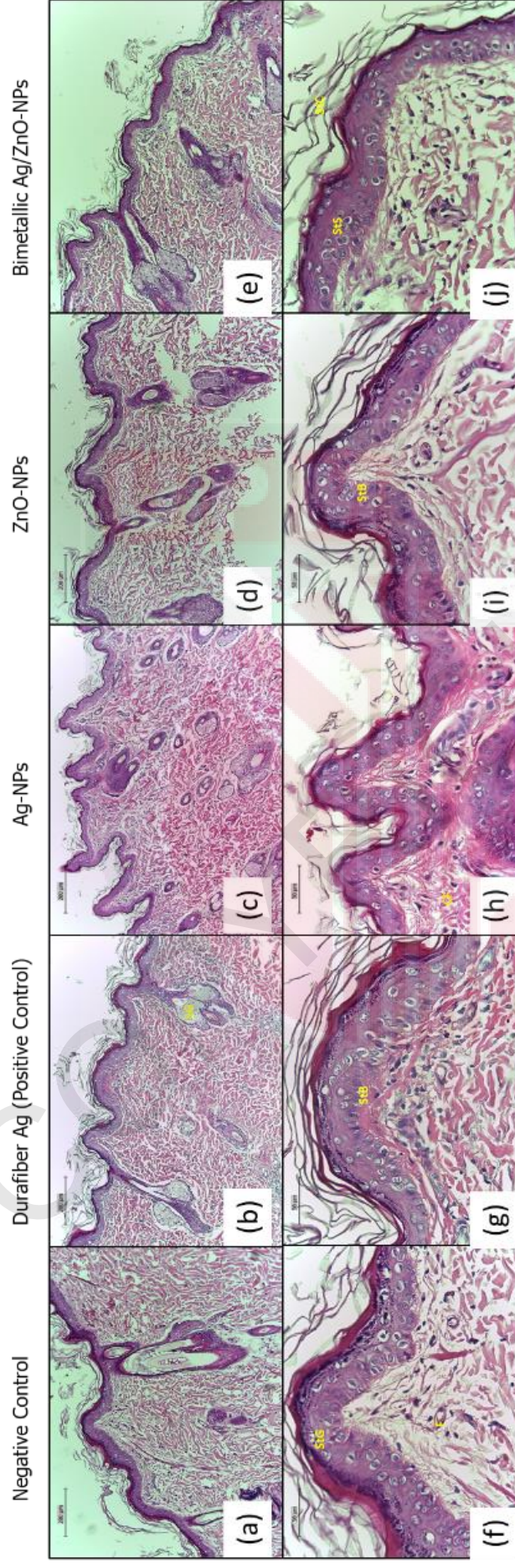


Figure 4.18: Histopathological evaluation of skin sections. Micrographs of H&E stained tissues of wounds treated with the developed wound dressing impregnated with synthesised NPs (Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs), commercial Durafiber-Ag as positive control as well as distilled water as negative control on the 20th day after induction of wounds ((a-e), 10x magnification and (f-j) 40x magnification).

4.5.3 Blood Biochemical and Haematological Analyses

The potential systemic toxicity of NPs is a significant concern, as their release of ions could induce inflammation or damage various organs through systemic circulation (Joudeh & Linke, 2022). To assess the safety of synthesised NPs, comprehensive blood biochemical and hematological analyses were conducted on Sprague-Dawley rats. Key parameters measured included Red Blood Cells (RBC), White Blood Cells (WBC), Hemoglobin (HGB), Platelets (PLT), and enzymes such as Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP), as well as Urea and Creatinine (Cre-enzyme) levels.

Figures 4.19 and 4.20 illustrate the results of these analyses performed on rats sacrificed after 20 days of treatment. Statistical analysis using one-way ANOVA followed by post hoc Tukey's test indicated no significant differences ($p > 0.05$) in the blood biochemical and hematological parameters between the rats treated with synthesised NPs (including Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs), those treated with the commercial Durafiber Ag (positive control), and those receiving untreated gauze (negative control).

The absence of significant deviations in these parameters indicates that the synthesised NPs did not induce systemic toxicity or adverse effects on the rats' health. The normal levels of RBC, WBC, HGB, PLT, and the various enzymes suggest that the cotton gauzes impregnated with synthesised NPs, regardless of type, do not compromise the overall health of the rats or affect organ function

adversely. This outcome aligns with Joudeh & Linke (2022), who emphasised the importance of thorough safety evaluations for NPs to ensure they do not inadvertently cause systemic toxicity.





Figure 4.19: Biochemical analysis; (a) ALT, (b) AST, (c) ALP, (d) Urea, and (e) Cre-enzyme.



Figure 4.20: Haematological analysis; (a) RBC, (b) WBC, (c) HGB, and (d) PLT

4.6 Summary

In this chapter, the results and discussions focus on the identification and characterisation of four main subclasses of polyphenolics, including phenolic acids, flavonoids, lignans, and xanthenes in SBH using UHPLC-MS/Q-TOF. These compounds were highlighted for their potent antioxidant, antimicrobial, and anti-inflammatory properties, which contribute not only to therapeutic benefits but also to their roles as reducing and capping agents in the synthesis of NPs. The successful synthesis of Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs with desired physicochemical properties was confirmed through various characterisation techniques. The antimicrobial activity of these synthesised NPs was demonstrated against both gram-positive and gram-negative bacteria, with all synthesised NPs showing significant efficacy. Notably, the monometallic Ag-NPs and ZnO-NPs exhibited selective cytotoxicity towards HaCaT cells, while remaining non-toxic to normal cells (3T3 fibroblasts and MRC5 lung fibroblasts). Remarkably, the synthesised bimetallic Ag/ZnO-NPs showed no cytotoxic effects on any of the tested cell lines, indicating their superior biocompatibility. In vivo studies further supported these findings, revealing that wound dressings treated with synthesised bimetallic Ag/ZnO-NPs facilitated the most efficient wound healing, with complete healing observed by day 20. This was corroborated by histopathological analysis, which showed improved tissue regeneration and minimal scarring. Moreover, blood biochemical and haematological analyses confirmed the absence of systemic toxicity, underscoring the safety and therapeutic potential of these synthesised NPs in wound healing applications.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The study successfully developed and characterised SBH-synthesised NPs, including Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs, as bioactive agents for wound dressing applications. These NPs were synthesised using SBH and characterised through multiple techniques to confirm their formation and properties. UV-Vis spectroscopy revealed distinct SPR bands at 414 nm for Ag-NPs, 334 nm for ZnO-NPs, and a broad band from 380 to 460 nm for bimetallic Ag/ZnO-NPs. Morphological analysis using FESEM and TEM showed spherical particles ranging from 15 to 50 nm with slight agglomeration. Elemental analysis via EDX verified the presence of silver and zinc oxide, while FT-IR confirmed functional groups such as O–H, C=C, aromatic C–H, and =C–H, which contribute to NP reduction and stabilisation. XRD analysis further validated their crystalline structures, with distinct diffraction peaks corresponding to the crystallographic planes of Ag-NPs and ZnO-NPs. The presence of both Ag and ZnO peaks in the bimetallic sample confirmed the successful formation of Ag/ZnO-NPs.

The synthesised NPs were then integrated into wound dressings, and SEM analysis demonstrated their attachment, deposition, and dispersion on cotton fabric. However, the distribution was irregular, forming clump-like patterns that indicated inhomogeneous dispersion. EDX analysis further supported these

findings by detecting Ag and Zn in fabrics treated with Ag-NPs, ZnO-NPs, and bimetallic Ag/ZnO-NPs, confirming their successful incorporation into the dressing material.

The antimicrobial properties of both the synthesised NPs and NP-treated gauzes were evaluated against *S. aureus* and *E. coli*, showing significant antibacterial activity with inhibition zones ranging from 12 to 20 mm. Biocompatibility tests revealed that Ag-NPs and ZnO-NPs exhibited inhibitory concentrations (IC_{50}) of 10 $\mu\text{g/mL}$ and 85 $\mu\text{g/mL}$ on HaCaT cells, respectively. Notably, bimetallic Ag/ZnO-NPs showed no toxicity effects on HaCaT cells up to 100 $\mu\text{g/mL}$. Furthermore, all synthesised NPs were non-toxic to normal 3T3 and MRC5 cells at concentrations up to 100 $\mu\text{g/mL}$, demonstrating their potential safety for wound healing applications.

The wound healing efficacy of these NP-treated gauzes was further confirmed through an in vivo study. The results indicated that gauzes impregnated with ZnO-NPs significantly accelerated wound healing by day 4 compared to untreated controls. This effect was further enhanced by bimetallic Ag/ZnO-NPs, which exhibited the most consistent and rapid healing, achieving complete wound closure by day 20. Histopathological analysis confirmed full tissue regeneration with no signs of damage, while blood tests showed no systemic toxicity. These findings highlight the synergistic benefits of bimetallic Ag/ZnO-NPs in promoting wound healing and ensuring biocompatibility.

Despite these promising results, certain limitations must be addressed. The study was conducted in a controlled laboratory setting, and further in vivo and clinical studies are necessary to validate the long-term efficacy and safety of SBH-NPs in real-world wound care applications. Additionally, while the antibacterial and wound healing properties of Ag-NPs and ZnO-NPs were examined, their long-term stability, cytotoxicity at higher concentrations, and interaction with various wound environments require further investigation.

5.2 Recommendations

Future research should focus on optimising the synthesis and formulation of SBH-NPs to enhance stability, biocompatibility, and efficacy while minimising potential toxicity. Large-scale production techniques must be explored to ensure consistency in synthesis and performance. Furthermore, standardised protocols for integrating these NPs into wound dressings should be developed, and their effectiveness should be tested in chronic wound models to assess clinical applicability.

From a commercialisation perspective, evaluating the economic feasibility of SBH-NPs-based wound dressings is crucial. Based on market prices, the estimated production cost for 150 gauzes per batch is RM343.87, translating to RM2.30 per gauze. With a 30% profit margin, the suggested commercial price is RM3.00 per piece, generating a profit of RM0.70 per gauze. Using one 350g bottle of SBH could yield an estimated net profit of RM3,675, highlighting the commercial viability of the product.

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APPENDICES

Appendix A: Preliminary Study

1.1 Particle size

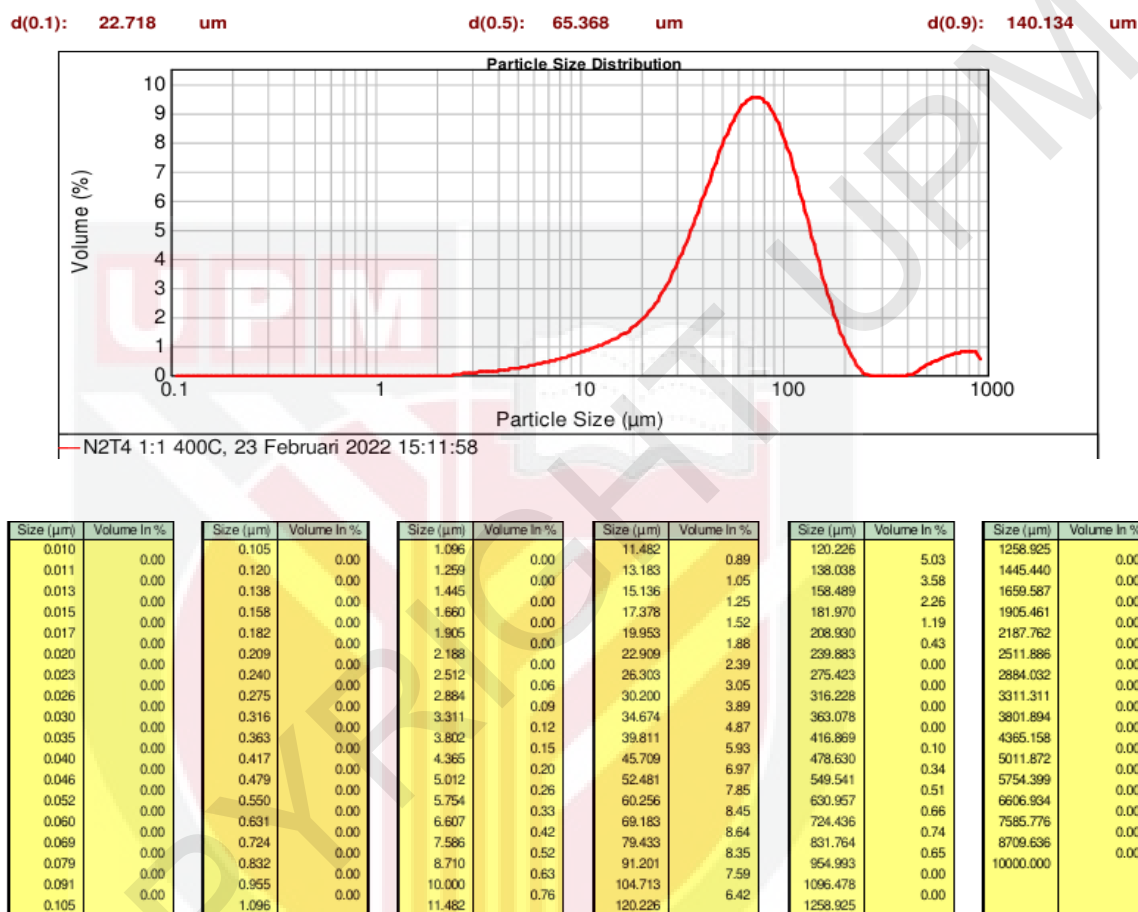
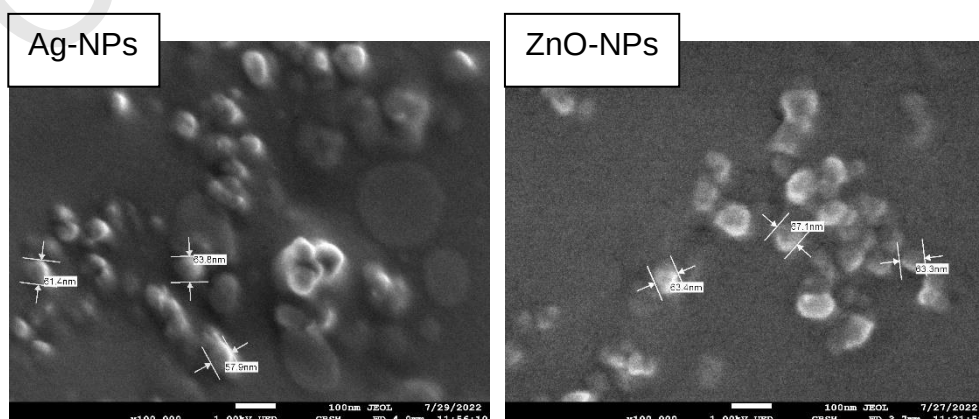


Figure A.1: Preliminary data for particle size of SBH's synthesised NPs.

1.2 FESEM-EDX analysis



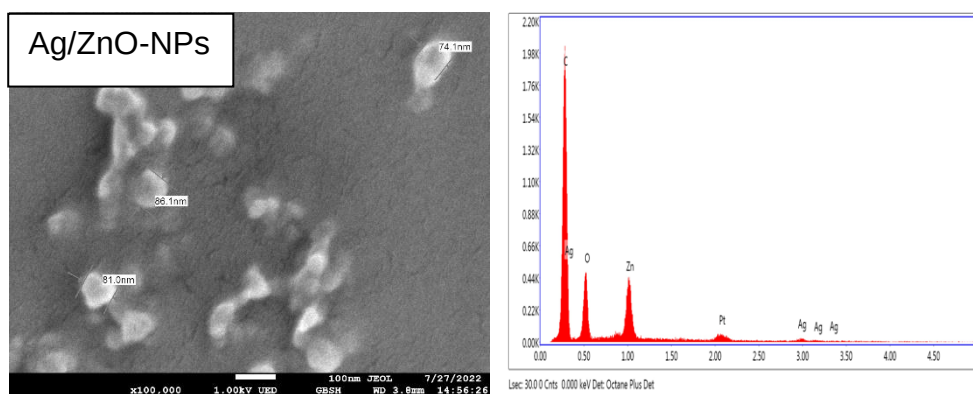


Figure A.2: Preliminary results for FESEM-EDX analysis of SBH's synthesised NPs

1.3 FTIR analysis

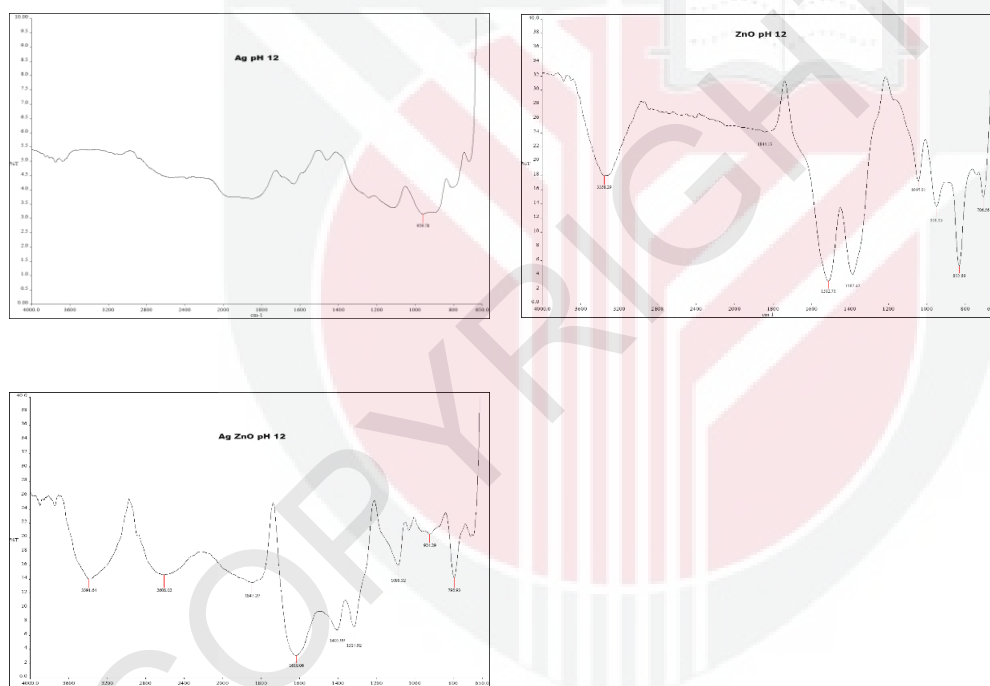


Figure A.3: Preliminary results for FTIR analysis of SBH's synthesised NPs

Appendix B: Statistical Data Evaluation

1.1 Statistical evaluation for antimicrobial analysis of SBH's synthesised NPs

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
NP\$Treatments	3	76.79	25.596	41.69	3.14e-05	***
Residuals	8	4.91	0.614			

Signif. codes:	0	***	0.001	**	0.01	*
	1				0.05	.
					0.1	

Figure B.1: ANOVA for antimicrobial analysis of SBH's synthesised NPs against gram-positive bacteria, *S. aureus*

\$ NP\$Treatments		diff	lwr
upr	p adj		
Ag/ZnO(5050)-Ag	37990 0.0013394	-3.8866667 -5.9353428	-1.8
Positive Control-Ag	15343 0.0310600	2.2666667 0.2179905	4.3
ZnO-Ag	72657 0.0030489	-3.4213333 -5.4700095	-1.3
Positive Control-Ag/ZnO(5050)	02010 0.0000528	6.1533333 4.1046572	8.2
ZnO-Ag/ZnO(5050)	14010 0.8835065	0.4653333 -1.5833428	2.5
ZnO-Positive Control	39324 0.0000939	-5.6880000 -7.7366762	-3.6

Figure B.2: Tukey multiple comparisons of means at 95% family-wise confidence level for antimicrobial analysis of SBH's synthesised NPs against gram-positive bacteria, *S. aureus*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)			
NP\$Treatments	3	34.31	11.437	35.62	5.63e-05	***		
Residuals	8	2.57	0.321					

Signif. codes:	0	***	0.001	**	0.01	*	0.05	.
'	1							0.1

Figure B.3: ANOVA for antimicrobial analysis of SBH's synthesised NPs against gram-negative bacteria, *E. coli*

\$`NP\$Treatments`		diff	lwr	
upr	p adj			
Ag/ZnO(5050)-Ag		-3.366667	-4.848348	-1.884
985	0.0003929			
Positive Control-Ag		-0.020000	-1.501682	1.461
682	0.9999684			
ZnO-Ag		-3.416667	-4.898348	-1.934
985	0.0003544			
Positive Control-Ag/ZnO(5050)		3.346667	1.864985	4.828
348	0.0004095			
ZnO-Ag/ZnO(5050)		-0.050000	-1.531682	1.431
682	0.9995087			
ZnO-Positive Control		-3.396667	-4.878348	-1.914
985	0.0003693			

Figure B.4: Tukey multiple comparisons of means at 95% family-wise confidence level for antimicrobial analysis of SBH's synthesised NPs against gram-negative bacteria, *E. coli*

1.2 Statistical evaluation for antimicrobial analysis of wound dressings impregnated with SBH's synthesised NPs

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Antimicrobe\$Treatments	3	44.76	14.919	8.143	0.00816
**					
Residuals	8	14.66	1.832		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

Figure B.5: ANOVA for antimicrobial analysis of wound dressings impregnated with SBH's synthesised NPs against gram-positive bacteria, *S. aureus*

\$`Antimicrobe\$Treatments`		diff	lwr	u
pr	p adj			
Ag/ZnO(5050)-Ag		-4.200000	-7.739187	-0.66080
13	0.0218146			
Durafiber Ag-Ag		-0.500000	-4.039187	3.03919
87	0.9672874			
ZnO-Ag		0.8533333	-2.6858653	4.39253
20	0.8648694			
Durafiber Ag-Ag/ZnO(5050)		3.700000	0.1608013	7.23919
87	0.0407655			
ZnO-Ag/ZnO(5050)		5.0533333	1.5141347	8.59253
20	0.0078790			

ZnO-Durafiber Ag	1.3533333	-2.1858653	4.89253
20	0.6298848		

Figure B.6: Tukey multiple comparisons of means at 95% family-wise confidence level for antimicrobial analysis of wound dressing impregnated with SBH's synthesised NPs against gram-positive bacteria, *S. aureus*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Antimicrobe\$Treatments	3	164.96	54.99	38.13	4.37e-05 ***
Residuals	8	11.53	1.44		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure B.7: ANOVA for antimicrobial analysis of wound dressings impregnated with SBH's synthesised NPs against gram-negative bacteria, *E. coli*

\$ Antimicrobe\$Treatments	diff	lwr	upr
p adj			
Ag/ZnO(5050)-Ag	-5.98	-9.119693	-2.8403066 0.
0013050			
Durafiber Ag-Ag	-0.28	-3.419693	2.8596934 0.
9912493			
ZnO-Ag	-8.64	-11.779693	-5.5003066 0.
0001002			
Durafiber Ag-Ag/ZnO(5050)	5.70	2.560307	8.8396934 0.
0017874			
ZnO-Ag/ZnO(5050)	-2.66	-5.799693	0.4796934 0.
0997290			
ZnO-Durafiber Ag	-8.36	-11.499693	-5.2203066 0.
0001273			

Figure B.8: Tukey multiple comparisons of means at 95% family-wise confidence level for antimicrobial analysis of wound dressings impregnated with SBH's synthesised NPs against gram-negative bacteria, *E. coli*

1.3 Statistical evaluation for cytotoxicity analysis of SBH's synthesised NPs

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
HaCaT\$treatment	2	3732	1865.9	3.774	0.0428 *
Residuals	18	8901	494.5		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Figure B.9: ANOVA for cytotoxicity analysis of SBH's synthesised NPs against HaCaT cell.

\$`HaCaT\$treatment`		diff	lwr	upr	p
adj					
Ag/ZnO-NPs-Ag-NPs	29.991286	-0.3438165	60.32639	0.0529	
450					
ZnO-NPs-Ag-NPs	26.178714	-4.1563879	56.51382	0.0978	
336					
ZnO-NPs-Ag/ZnO-NPs	-3.812571	-34.1476736	26.52253	0.9450	
351					

Figure B.10: Tukey multiple comparisons of means at 95% family-wise confidence level for cytotoxicity analysis of SBH's synthesised NPs against HaCaT cell

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
mouse\$treatment	2	67.5	33.77	0.215	0.809
Residuals	18	2832.0	157.33		

Figure B.11: ANOVA for cytotoxicity analysis of SBH's synthesised NPs against 3T3 cell

\$`mouse\$treatment`		diff	lwr	upr	p	a
adj						
Ag/ZnO-NPs-Ag-NPs	-3.4262857	-20.53771	13.68514	0.86696		
62						
ZnO-NPs-Ag-NPs	-4.0935714	-21.20500	13.01786	0.81624		
54						
ZnO-NPs-Ag/ZnO-NPs	-0.6672857	-17.77871	16.44414	0.99455		
56						

Figure B.12: Tukey multiple comparisons of means at 95% family-wise confidence level for cytotoxicity analysis of SBH's synthesised NPs against 3T3 cell

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
MRC5\$treatment	2	403.2	201.61	2.336	0.125
Residuals	18	1553.3	86.29		

Figure B.13: ANOVA for cytotoxicity analysis of SBH's synthesised NPs against MRC5 cell

		diff	lwr	upr	p a
dj					
Ag/ZnO-NPs-Ag-NPs	70	-4.911714	-17.58420	7.760769	0.59283
ZnO-NPs-Ag-NPs	94	-10.721000	-23.39348	1.951483	0.10594
ZnO-NPs-Ag/ZnO-NPs	10	-5.809286	-18.48177	6.863198	0.48546

Figure B.14: Tukey multiple comparisons of means at 95% family-wise confidence level for cytotoxicity analysis of SBH's synthesised NPs against MRC5 cell

1.4 Statistical evaluation for wound closure analysis

Response: Closure\$Four					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Closure\$Treatments	4	1856.8	464.21	3.2704	0.01778 *
Residuals	55	7806.8	141.94		

Signif. codes:	0	'***'	0.001	'**'	0.01
	'			'*	0.05
					'.'
					0.1

Figure B.15: ANOVA for wound closure analysis after day 4 post wounding

		diff	lwr	upr	p adj
Ag/ZnO (50:50)-Ag	070921	-4.646667	-18.36425439	9.070921	0.8736457
Control-Ag	748421	9.0308333	-4.68675439	22.748421	0.3525788
Durafiber-Ag-Ag	971754	0.2541667	-13.46342105	13.971754	0.9999983
ZnO-Ag	443421	-7.2741667	-20.99175439	6.443421	0.5697117
Control-Ag/ZnO (50:50)	395088	13.6775000	-0.04008772	27.395088	0.0510235
Durafiber-Ag-Ag/ZnO (50:50)	618421	4.9008333	-8.81675439	18.618421	0.8507794
ZnO-Ag/ZnO (50:50)	090088	-2.6275000	-16.34508772	11.090088	0.9826855
Durafiber-Ag-Control	940921	-8.7766667	-22.49425439	4.940921	0.3814881
ZnO-Control	587412	-16.3050000	-30.02258772	-2.587412	0.0121693
ZnO-Durafiber-Ag	189254	-7.5283333	-21.24592105	6.189254	0.5365895

Figure B.16: Tukey multiple comparisons of means at 95% family-wise confidence level for wound closure analysis after day 4 post wounding

Response: Closure\$Eight						
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Closure\$Treatments	4	255.05	63.763	1.2234	0.3115	
Residuals	55	2866.68	52.122			

Figure B.17: ANOVA for wound closure analysis after day 8 post wounding

\$` Closure\$Treatments`				diff	lwr	
upr	p	adj				
Ag/ZnO (50:50)-Ag	0.017	0.8705387		-2.8375000	-11.150017	5.475
Control-Ag	0.351	0.9064345		2.5658333	-5.746684	10.878
Durafiber-Ag-Ag	0.517	0.9357110		2.2950000	-6.017517	10.607
ZnO-Ag	0.017	0.9937187		-1.2175000	-9.530017	7.095
Control-Ag/ZnO (50:50)	0.851	0.3654123		5.4033333	-2.909184	13.715
Durafiber-Ag-Ag/ZnO (50:50)	0.017	0.4178266		5.1325000	-3.180017	13.445
ZnO-Ag/ZnO (50:50)	0.517	0.9815367		1.6200000	-6.692517	9.932
Durafiber-Ag-Control	0.684	0.9999833		-0.2708333	-8.583351	8.041
ZnO-Control	0.184	0.7022185		-3.7833333	-12.095851	4.529
ZnO-Durafiber-Ag	0.017	0.7560275		-3.5125000	-11.825017	4.800

Figure B.18: Tukey multiple comparisons of means at 95% family-wise confidence level for wound closure analysis after day 8 post wounding

Response: Closure\$Twelve						
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Closure\$Treatments	4	29.57	7.3913	0.4061	0.8034	
Residuals	55	1000.94	18.1990			

Figure B.19: ANOVA for wound closure analysis after day 12 post wounding

\$ Closure\$Treatments		diff	lwr	u
pr	p adj			
Ag/ZnO (50:50)-Ag		-0.20250000	-5.114379	4.7093
79	0.9999574			
Control-Ag		1.61833333	-3.293546	6.5302
13	0.8843295			
Durafiber-Ag-Ag		0.52333333	-4.388546	5.4352
13	0.9981699			
ZnO-Ag		-0.28166667	-5.193546	4.6302
13	0.9998414			
Control-Ag/ZnO (50:50)		1.82083333	-3.091046	6.7327
13	0.8330014			
Durafiber-Ag-Ag/ZnO (50:50)		0.72583333	-4.186046	5.6377
13	0.9935015			
ZnO-Ag/ZnO (50:50)		-0.07916667	-4.991046	4.8327
13	0.9999990			
Durafiber-Ag-Control		-1.09500000	-6.006879	3.8168
79	0.9697818			
ZnO-Control		-1.90000000	-6.811879	3.0118
79	0.8104223			
ZnO-Durafiber-Ag		-0.80500000	-5.716879	4.1068
79	0.9903590			

Figure B.20: Tukey multiple comparisons of means at 95% family-wise confidence level for wound closure analysis after day 12 post wounding

Response: Closure\$Sixteen						
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Closure\$Treatments	4	1.339	0.33468	0.4358	0.7822	
Residuals	55	42.237	0.76795			

Figure B.21: ANOVA for wound closure analysis after day 16 post wounding

\$ Closure\$Treatments		diff	lwr	u
upr	p adj			
Ag/ZnO (50:50)-Ag		-0.35750000	-1.3664993	0.651
4993	0.8545571			
Control-Ag		0.03000000	-0.9789993	1.038
9993	0.9999884			
Durafiber-Ag-Ag		-0.17500000	-1.1839993	0.833
9993	0.9880582			
ZnO-Ag		-0.26416667	-1.2731660	0.744
8326	0.9464901			
Control-Ag/ZnO (50:50)		0.38750000	-0.6214993	1.396
4993	0.8144007			
Durafiber-Ag-Ag/ZnO (50:50)		0.18250000	-0.8264993	1.191
4993	0.9860194			
ZnO-Ag/ZnO (50:50)		0.09333333	-0.9156660	1.102
3326	0.9989487			

Durafiber-Ag-Control	-0.20500000	-1.2139993	0.803
9993 0.9784670			
ZnO-Control	-0.29416667	-1.3031660	0.714
8326 0.9226418			
ZnO-Durafiber-Ag	-0.08916667	-1.0981660	0.919
8326 0.9991216			

Figure B.22: Tukey multiple comparisons of means at 95% family-wise confidence level for wound closure analysis after day 16 post wounding

1.5 Statistical evaluation for blood biochemical and haematology analysis

Response: Blood\$RBC					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blood\$Treatments	4	5.0414	1.2604	1.117	0.3707
Residuals	25	28.2078	1.1283		

Figure B.23: ANOVA for RBC analysis

\$` Blood\$Treatments`		diff	1
wr	upr		
Ag/ZnO-NPs (50:50)-Ag-NPs		0.88833333	-0.91277
00 2.6894366			
Durafiber Ag-Ag-NPs		0.68500000	-1.11610
33 2.4861033			
Negative Control-Ag-NPs		0.81000000	-0.99110
33 2.6111033			
ZnO-NPs-Ag-NPs		-0.06166667	-1.86277
00 1.7394366			
Durafiber Ag-Ag/ZnO-NPs (50:50)		-0.20333333	-2.00443
66 1.5977700			
Negative Control-Ag/ZnO-NPs (50:50)		-0.07833333	-1.87943
66 1.7227700			
ZnO-NPs-Ag/ZnO-NPs (50:50)		-0.95000000	-2.75110
33 0.8511033			
Negative Control-Durafiber Ag		0.12500000	-1.67610
33 1.9261033			
ZnO-NPs-Durafiber Ag		-0.74666667	-2.54777
00 1.0544366			
ZnO-NPs-Negative Control		-0.87166667	-2.67277
00 0.9294366			
	p adj		
Ag/ZnO-NPs (50:50)-Ag-NPs	0.6035714		
Durafiber Ag-Ag-NPs	0.7961465		
Negative Control-Ag-NPs	0.6812462		
ZnO-NPs-Ag-NPs	0.9999751		
Durafiber Ag-Ag/ZnO-NPs (50:50)	0.9972128		
Negative Control-Ag/ZnO-NPs (50:50)	0.9999354		
ZnO-NPs-Ag/ZnO-NPs (50:50)	0.5419319		
Negative Control-Durafiber Ag	0.9995871		

ZnO-NPs-Durafiber Ag	0.7414803
ZnO-NPs-Negative Control	0.6202449

Figure B.24: Tukey multiple comparisons of means at 95% family-wise confidence level for RBC analysis

Response: Blood\$WBC						
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Blood\$Treatments	4	12.302	3.0755	0.4398	0.7786	
Residuals	25	174.812	6.9925			

Figure B.25: ANOVA for WBC analysis

\$ Blood\$Treatments			diff	lwr
upr	p adj			
Ag/ZnO-NPs (50:50)-Ag-NPs		0.9833333	-3.500397	
5.467064 0.9661627				
Durafiber Ag-Ag-NPs		1.6000000	-2.883730	
6.083730 0.8305883				
Negative Control-Ag-NPs		-0.1000000	-4.583730	
4.383730 0.9999955				
ZnO-NPs-Ag-NPs		0.8833333	-3.600397	
5.367064 0.9770362				
Durafiber Ag-Ag/ZnO-NPs (50:50)		0.6166667	-3.867064	
5.100397 0.9940319				
Negative Control-Ag/ZnO-NPs (50:50)		-1.0833333	-5.567064	
3.400397 0.9523596				
ZnO-NPs-Ag/ZnO-NPs (50:50)		-0.1000000	-4.583730	
4.383730 0.9999955				
Negative Control-Durafiber Ag		-1.7000000	-6.183730	
2.783730 0.7979327				
ZnO-NPs-Durafiber Ag		-0.7166667	-5.200397	
3.767064 0.9894373				
ZnO-NPs-Negative Control		0.9833333	-3.500397	
5.467064 0.9661627				

Figure B.26: Tukey multiple comparisons of means at 95% family-wise confidence level for WBC analysis

Response: Blood\$HGB						
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Blood\$Treatments	4	20.095	5.0238	1.6392	0.1957	
Residuals	25	76.618	3.0647			

Figure B.27: ANOVA for HGB analysis

\$`Blood\$Treatments`		diff	lwr
upr			
Ag/ZnO-NPs (50:50)-Ag-NPs	4.4517210	1.4833333	-1.485054
Durafiber Ag-Ag-NPs	3.3683877	0.4000000	-2.568388
Negative Control-Ag-NPs	4.2183877	1.2500000	-1.718388
ZnO-NPs-Ag-NPs	2.2183877	-0.7500000	-3.718388
Durafiber Ag-Ag/ZnO-NPs (50:50)	1.8850543	-1.0833333	-4.051721
Negative Control-Ag/ZnO-NPs (50:50)	2.7350543	-0.2333333	-3.201721
ZnO-NPs-Ag/ZnO-NPs (50:50)	0.7350543	-2.2333333	-5.201721
Negative Control-Durafiber Ag	3.8183877	0.8500000	-2.118388
ZnO-NPs-Durafiber Ag	1.8183877	-1.1500000	-4.118388
ZnO-NPs-Negative Control	0.9683877	-2.0000000	-4.968388
		p adj	
Ag/ZnO-NPs (50:50)-Ag-NPs		0.5918564	
Durafiber Ag-Ag-NPs		0.9944811	
Negative Control-Ag-NPs		0.7305362	
ZnO-NPs-Ag-NPs		0.9443726	
Durafiber Ag-Ag/ZnO-NPs (50:50)		0.8190030	
Negative Control-Ag/ZnO-NPs (50:50)		0.9993247	
ZnO-NPs-Ag/ZnO-NPs (50:50)		0.2089431	
Negative Control-Durafiber Ag		0.9151384	
ZnO-NPs-Durafiber Ag		0.7852197	
ZnO-NPs-Negative Control		0.3048545	

Figure B.28: Tukey multiple comparisons of means at 95% family-wise confidence level for HGB analysis

Response: Blood\$PLT					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blood\$Treatments	4	153793	38448	1.268	0.3088
Residuals	25	758035	30321		

Figure B.29: ANOVA for PLT analysis

\$`Blood\$Treatments`		diff
lwr	upr	
Ag/ZnO-NPs (50:50)-Ag-NPs	278 219.0894	-76.1666667 -371.42
Durafiber Ag-Ag-NPs	945 421.9228	126.6666667 -168.58

Negative Control-Ag-NPs	86.8333333	-208.42
278 382.0894		
ZnO-NPs-Ag-NPs	-0.3333333	-295.58
945 294.9228		
Durafiber Ag-Ag/ZnO-NPs (50:50)	202.8333333	-92.42
278 498.0894		
Negative Control-Ag/ZnO-NPs (50:50)	163.0000000	-132.25
611 458.2561		
ZnO-NPs-Ag/ZnO-NPs (50:50)	75.8333333	-219.42
278 371.0894		
Negative Control-Durafiber Ag	-39.8333333	-335.08
945 255.4228		
ZnO-NPs-Durafiber Ag	-127.0000000	-422.25
611 168.2561		
ZnO-NPs-Negative Control	-87.1666667	-382.42
278 208.0894		
	p adj	
Ag/ZnO-NPs (50:50)-Ag-NPs	0.9402587	
Durafiber Ag-Ag-NPs	0.7171427	
Negative Control-Ag-NPs	0.9073701	
ZnO-NPs-Ag-NPs	1.0000000	
Durafiber Ag-Ag/ZnO-NPs (50:50)	0.2869903	
Negative Control-Ag/ZnO-NPs (50:50)	0.4982639	
ZnO-NPs-Ag/ZnO-NPs (50:50)	0.9411493	
Negative Control-Durafiber Ag	0.9944563	
ZnO-NPs-Durafiber Ag	0.7152145	
ZnO-NPs-Negative Control	0.9062050	

Figure B.30: Tukey multiple comparisons of means at 95% family-wise confidence level for PLT analysis

Response: Blood\$ALT						
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Blood\$Treatments	4	557.13	139.28	1.2273	0.3245	
Residuals	25	2837.17	113.49			

Figure B.31: ANOVA for ALT analysis

\$ Blood\$Treatments			
	diff	lwr	upr
Ag/ZnO-NPs (50:50)-Ag-NPs	-11.5000000	-29.5632	6.563283
8 6.563283			
Durafiber Ag-Ag-NPs	-6.1666667	-24.2299	11.896617
5 11.896617			
Negative Control-Ag-NPs	-7.0000000	-25.0632	11.063283
8 11.063283			
ZnO-NPs-Ag-NPs	-11.8333333	-29.8966	6.229950
2 6.229950			
Durafiber Ag-Ag/ZnO-NPs (50:50)	5.3333333	-12.7299	23.396617
5 23.396617			

```

Negative Control-Ag/ZnO-NPs (50:50)  4.5000000 -13.5632
8 22.563283
ZnO-NPs-Ag/ZnO-NPs (50:50)         -0.3333333 -18.3966
2 17.729950
Negative Control-Durafiber Ag       -0.8333333 -18.8966
2 17.229950
ZnO-NPs-Durafiber Ag               -5.6666667 -23.7299
5 12.396617
ZnO-NPs-Negative Control            -4.8333333 -22.8966
2 13.229950

                                p adj
Ag/ZnO-NPs (50:50)-Ag-NPs          0.3586764
Durafiber Ag-Ag-NPs                 0.8516743
Negative Control-Ag-NPs             0.7850473
ZnO-NPs-Ag-NPs                     0.3312643
Durafiber Ag-Ag/ZnO-NPs (50:50)    0.9061695
Negative Control-Ag/ZnO-NPs (50:50) 0.9470153
ZnO-NPs-Ag/ZnO-NPs (50:50)         0.9999979
Negative Control-Durafiber Ag       0.9999184
ZnO-NPs-Durafiber Ag               0.8859602
ZnO-NPs-Negative Control            0.9323430

```

Figure B.32: Tukey multiple comparisons of means at 95% family-wise confidence level for ALT analysis

```

Response: Blood$ALP
      Df Sum Sq Mean Sq F value Pr(>F)
Blood$Treatments  4  12286   3071.6    2.3325 0.08347 .
Residuals        25  32923   1316.9
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1
                  ' ' 1

```

Figure B.33: ANOVA for ALP analysis

```

$`Blood$Treatments`
                                diff      1w
r      upr
Ag/ZnO-NPs (50:50)-Ag-NPs      -27.833333 -89.3652
8 33.698616
Durafiber Ag-Ag-NPs           -12.166667 -73.6986
2 49.365283
Negative Control-Ag-NPs        -3.000000 -64.5319
5 58.531949
ZnO-NPs-Ag-NPs                -55.166667 -116.6986
2  6.365283
Durafiber Ag-Ag/ZnO-NPs (50:50)  15.666667 -45.8652
8 77.198616
Negative Control-Ag/ZnO-NPs (50:50) 24.833333 -36.6986
2 86.365283
ZnO-NPs-Ag/ZnO-NPs (50:50)     -27.333333 -88.8652
8 34.198616

```

```

Negative Control-Durafiber Ag      9.166667 -52.3652
8 70.698616
ZnO-NPs-Durafiber Ag             -43.000000 -104.5319
5 18.531949
ZnO-NPs-Negative Control         -52.166667 -113.6986
2  9.365283

                                p adj
Ag/ZnO-NPs (50:50)-Ag-NPs        0.6766476
Durafiber Ag-Ag-NPs              0.9767280
Negative Control-Ag-NPs          0.9998983
ZnO-NPs-Ag-NPs                   0.0943970
Durafiber Ag-Ag/ZnO-NPs (50:50)  0.9428834
Negative Control-Ag/ZnO-NPs (50:50) 0.7595089
ZnO-NPs-Ag/ZnO-NPs (50:50)      0.6908913
Negative Control-Durafiber Ag    0.9919065
ZnO-NPs-Durafiber Ag            0.2715585
ZnO-NPs-Negative Control        0.1251294

```

Figure B.34: Tukey multiple comparisons of means at 95% family-wise confidence level for ALP analysis

```

Response: Blood$AST
              Df Sum Sq Mean Sq F value Pr(>F)
Blood$Treatments 4  1656.5   414.13    3.6022 0.01885 *
Residuals       25  2874.2   114.97
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1
                  ' ' 1

```

Figure B.35: ANOVA for AST analysis

```

$`Blood$Treatments`
                                diff      1wr
upr
Ag/ZnO-NPs (50:50)-Ag-NPs        2.500000 -15.68069
20.6806851
Durafiber Ag-Ag-NPs              -9.166667 -27.34735
9.0140184
Negative Control-Ag-NPs          -7.833333 -26.01402
10.3473518
ZnO-NPs-Ag-NPs                  -18.500000 -36.68069
-0.3193149
Durafiber Ag-Ag/ZnO-NPs (50:50) -11.666667 -29.84735
6.5140184
Negative Control-Ag/ZnO-NPs (50:50) -10.333333 -28.51402
7.8473518
ZnO-NPs-Ag/ZnO-NPs (50:50)      -21.000000 -39.18069
-2.8193149
Negative Control-Durafiber Ag     1.333333 -16.84735
19.5140184
ZnO-NPs-Durafiber Ag            -9.333333 -27.51402
8.8473518

```

ZnO-NPs-Negative Control	-10.666667	-28.84735
7.5140184		
	p adj	
Ag/ZnO-NPs (50:50)-Ag-NPs	0.9940362	
Durafiber Ag-Ag-NPs	0.5837597	
Negative Control-Ag-NPs	0.7139740	
ZnO-NPs-Ag-NPs	0.0446866	
Durafiber Ag-Ag/ZnO-NPs (50:50)	0.3510413	
Negative Control-Ag/ZnO-NPs (50:50)	0.4698924	
ZnO-NPs-Ag/ZnO-NPs (50:50)	0.0178809	
Negative Control-Durafiber Ag	0.9994864	
ZnO-NPs-Durafiber Ag	0.5672355	
ZnO-NPs-Negative Control	0.4387040	

Figure B.36: Tukey multiple comparisons of means at 95% family-wise confidence level for AST analysis

Response: Blood\$Urea						
	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Blood\$Treatments	4	3.277	0.81915	0.4351	0.782	
Residuals	25	47.068	1.88274			

Figure B.37: ANOVA for urea analysis

\$ Blood\$Treatments`			diff	1w
r	upr			
Ag/ZnO-NPs (50:50)-Ag-NPs		0.69666667	-1.62992	
0 3.023254				
Durafiber Ag-Ag-NPs		-0.29166667	-2.61825	
4 2.034920				
Negative Control-Ag-NPs		0.09666667	-2.22992	
0 2.423254				
ZnO-NPs-Ag-NPs		0.31666667	-2.00992	
0 2.643254				
Durafiber Ag-Ag/ZnO-NPs (50:50)		-0.98833333	-3.31492	
0 1.338254				
Negative Control-Ag/ZnO-NPs (50:50)		-0.60000000	-2.92658	
7 1.726587				
ZnO-NPs-Ag/ZnO-NPs (50:50)		-0.38000000	-2.70658	
7 1.946587				
Negative Control-Durafiber Ag		0.38833333	-1.93825	
4 2.714920				
ZnO-NPs-Durafiber Ag		0.60833333	-1.71825	
4 2.934920				
ZnO-NPs-Negative Control		0.22000000	-2.10658	
7 2.546587				
	p adj			
Ag/ZnO-NPs (50:50)-Ag-NPs	0.9017842			
Durafiber Ag-Ag-NPs	0.9958195			
Negative Control-Ag-NPs	0.9999462			
ZnO-NPs-Ag-NPs	0.9942655			

Durafiber Ag-Ag/ZnO-NPs (50:50)	0.7242954
Negative Control-Ag/ZnO-NPs (50:50)	0.9403218
ZnO-NPs-Ag/ZnO-NPs (50:50)	0.9885422
Negative Control-Durafiber Ag	0.9875704
ZnO-NPs-Durafiber Ag	0.9374435
ZnO-NPs-Negative Control	0.9986039

Figure B.38: Tukey multiple comparisons of means at 95% family-wise confidence level for urea analysis

Response: Blood\$Cre.enzyme					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Blood\$Treatments	4	37.80	9.450	0.7299	0.58
Residuals	25	323.67	12.947		

Figure B.39: ANOVA for Cre-enzyme analysis

\$ Blood\$Treatments		diff
lwr	upr	
Ag/ZnO-NPs (50:50)-Ag-NPs		-1.666667e-01 -6.267
698 5.934365		
Durafiber Ag-Ag-NPs		-1.500000e+00 -7.601
031 4.601031		
Negative Control-Ag-NPs		-1.666667e-01 -6.267
698 5.934365		
ZnO-NPs-Ag-NPs		2.000000e+00 -4.101
031 8.101031		
Durafiber Ag-Ag/ZnO-NPs (50:50)		-1.333333e+00 -7.434
365 4.767698		
Negative Control-Ag/ZnO-NPs (50:50)		5.329071e-15 -6.101
031 6.101031		
ZnO-NPs-Ag/ZnO-NPs (50:50)		2.166667e+00 -3.934
365 8.267698		
Negative Control-Durafiber Ag		1.333333e+00 -4.767
698 7.434365		
ZnO-NPs-Durafiber Ag		3.500000e+00 -2.601
031 9.601031		
ZnO-NPs-Negative Control		2.166667e+00 -3.934
365 8.267698		
	p adj	
Ag/ZnO-NPs (50:50)-Ag-NPs	0.9999899	
Durafiber Ag-Ag-NPs	0.9493822	
Negative Control-Ag-NPs	0.9999899	
ZnO-NPs-Ag-NPs	0.8690722	
Durafiber Ag-Ag/ZnO-NPs (50:50)	0.9665851	
Negative Control-Ag/ZnO-NPs (50:50)	1.0000000	
ZnO-NPs-Ag/ZnO-NPs (50:50)	0.8329926	
Negative Control-Durafiber Ag	0.9665851	
ZnO-NPs-Durafiber Ag	0.4607845	
ZnO-NPs-Negative Control	0.8329926	

Figure B.40: Tukey multiple comparisons of means at 95% family-wise confidence level for Cre-enzyme analysis

Appendix C: Instruments and Analysis

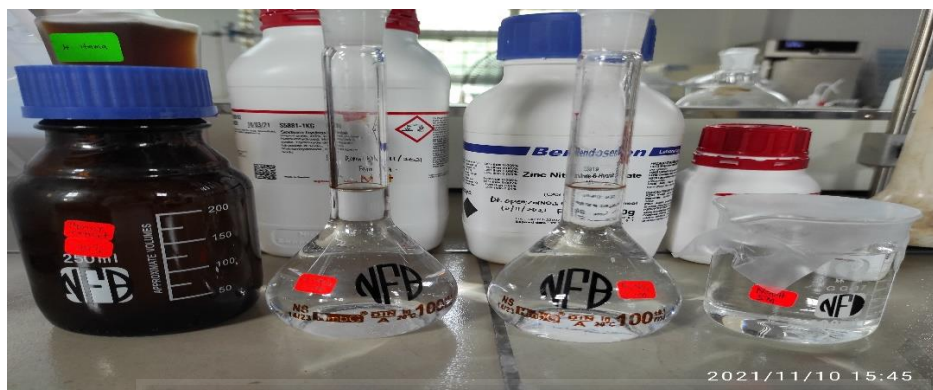


Figure C.1: Chemicals used for the synthesis of SBH's synthesised NPs



Figure C.2: FESEM-EDX analysis



Figure C.3: UV-vis analysis



Figure C.4: Preparation of circular wound on the dorsum of rats



Figure C.5: Preparation of treated gauzes for daily wound dressing



Figure C.6: Blood taken for biochemical and haematology analysis

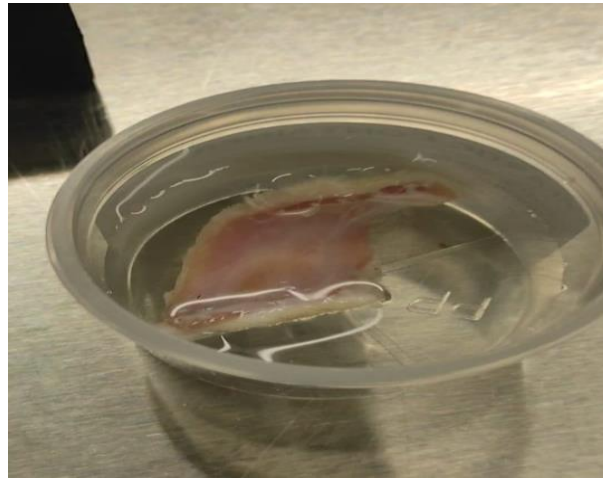


Figure C.7: Skin for histopathology analysis (stored in Formaline)



Figure C.8: Histopathological analysis

Appendix D: Calculations

1.1 Preparation of 10% honey (w/v) solution.

= 10 g in 100 mL of distilled water

1.2 Preparation of 5 M NaOH solution

Mass (g) = (molecular weight of NaOH x M x volume) / 1000

$$= (40 \times 5 \times 100) / 1000$$

= 20 g in 100 mL distilled water.

1.3 Preparation of synthesised NPs

a) Ag-NPs

10g AgNO₃ in 100 mL of distilled water.

b) ZnO-NPs

10g Zn(NO₃)₂ in 100 mL of distilled water.

c) Bimetallic Ag/ZnO-NPs

5g AgNO₃ + 5g Zn(NO₃)₂ in 100 mL of distilled water.

BIODATA OF STUDENT

Norfarina binti Bahari holds a Bachelor of Science (Hons) in Petroleum Chemistry, obtained in 2005, and furthered her studies with a Master of Science in Chemistry, completed in 2007. Her professional journey commenced in 2008 as a lecturer at Terengganu Advanced Technical University College (TaTiUC). In 2009, she transitioned her career to the realm of agricultural research by joining the Malaysia Agricultural Research and Development Institute (MARDI) as a Research Officer. Currently, she is pursuing a PhD under the sponsorship of MARDI at Universiti Putra Malaysia (UPM), demonstrating her unwavering commitment to academic growth and research excellence. Beyond her professional endeavors, Norfarina is a devoted wife to Mohd Shukry bin Hassan Basri and a loving mother to their son, Affan Hadif bin Mohd Shukry. Despite her responsibilities, she maintains a humble and aspiring spirit, driven by her passion for knowledge and a desire to make a meaningful impact through her research.

LIST OF PUBLICATIONS

Journals:

Bahari, N., Hashim, N., Abdan, K., Md Akim, A., Maringgal, B., & Al-Shdifat, L. (2025). Green-Synthesised Silver and Zinc Oxide Nanoparticles from Stingless Bee Honey: Morphological Characterisation, Antimicrobial Action, and Cytotoxic Assessment. *Chemosphere*, 370, 143961. <https://doi.org/10.1016/j.chemosphere.2024.143961> (WoS; Q1; IF: 8.1).

Bahari, N., Hashim, N., Abdan, K., Akim, A., Maringgal, B., & Al-Shdifat, L. (2023). Role of Honey as a Bifunctional Reducing and Capping / Stabilising Agent: Application for Silver and Zinc Oxide Nanoparticles. *Nanomaterials*, 13, 1244. <https://doi.org/10.3390/nano13071244> (WoS; Q1; IF: 5.3).

Bahari, N., Hashim, N., Md Akim, A., & Maringgal, B. (2022). Recent Advances in Honey-Based Nanoparticles for Wound Dressing: A Review. *Nanomaterials*, 12(15), 2560. <https://doi.org/10.3390/nano12152560> (WoS; Q1; IF: 5.3).

Patent:

Hashim, N., **Bahari, N.**, Rozman, A.S., Md Akim, A., Abdan, K., Maringgal, B., & Al-Shdifat, L. (2024). Article Comprising Stingless Bee Honey-Based Nanoparticle and Stingless Bee Honey Extract for Improving Wound Healing Rate and Method Thereof. (Filing No: PI 2024005945).

