DEVELOPMENT OF ANTIBACTERIAL CHITOSAN-BASED WOUND DRESSING INCORPORATED WITH ZNO/CUO NANOPARTICLES USING *CALOTROPIS GIGANTEA*

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UNIVERSITI SAINS MALAYSIA

2023

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

June 2023

ACKNOWLEDGEMENT

In the name of Mighty God of Heaven, the Most Gracious, the Most Merciful. All praises and gratitude belong to Jesus Christ, the sources of my strength and pillar in completing the thesis throughout this semester despite Covid-19 challenges. First and foremost, I wish to express my sincerest gratitude to my dedicated supervisor, Ts. Dr. Rabiatul Basria S.M.N. Mydin, for her continuous support, her patience and she consistently guide me in the right direction whenever she thought I needed it. I would also like to express my thanks to my great co-supervisors, Prof. Ir. Dr. Srimala Sreekantan for guiding me especially in writing papers and ideas despite her tight schedules. May Mighty God grants abundant blessings for these great people. Besides, I would also like to take this opportunity to express my thanks to those people who have helped me throughout my research period, especially from Animal Research & Service Centre (ARASC), Advanced Medical & Dental Institute (AMDI), and School of Materials and Mineral Resources Engineering (SMMRE) in providing services and facilities for this research to be well conducted. I will not also forget the help obtained from Dr. Nor Azlina Binti Khalil, Dr. Nor Aini Saidin, Dr. Isma Suzyta Ismail and staff ARASC, which without them, I may not be able to even perform animal work. I would also like to extend my gratitude to seniors (Nor Hazliana and Wan Nuramiera) and juniors which involved in my research. Lastly, I would like to express my deepest gratitude to my parents (Mr. Govindasamy and Mrs. Meenachy), beloved wife (Viel Anak Sulis), beautiful daughters (Ellil Hannah and Kezia Mounashree), family members, friends and colleagues for their unfailing support and prayers through the process of researching and writing this thesis.

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LIST OF UNITS AND SYMBOLS

H_2O_2	Hydrogen peroxide
·OH	Hydroxyl radicals
mL	Milliliter
µM	Micrometre
µL	Microliter
%	Percentage
rpm	Revolutions per minute
nm	Nanometre
Cu^{2+}	Copper ions
Zn^{2+}	Zinc ions
mm/min	Millimetres per minute
λ	X-ray wavelength
θ	Bragg diffraction angle
FWHM	Full-width at half-maximum
d	Crystallite size
K	Shape factor
kV	Kilovolt
mA	Milliamp
h	hour
v/v	Volume per volume
wt.%	Weight percentage
g/cm^3	Density
Ɵ	Contact angle
mg/mL	Milligrams per milliliter
CFU/mL	Colony Forming Unit per millilitre
mg/kg	Milligrams per kilogram
R_M	Cell migration rate

LIST OF ABBREVIATIONS

C. gigantea	*Calotropis gigantea*
ISO	International Organisation for Standardisation
SSA	Specific surface area
MW	Molecular weight
DMSO	Dimethyl sulfoxide
MDR	Multi drug resistant
S. aureus	*Staphylococcus aureus*
K.pneumoniae	*Klebsiella pneumonia*
E. coli	*Escherichia coli*
MRSA	methicillin-resistant *Staphylococcus aureus*
P. aeruginosa	*Pseudomonas aeruginosa*
ATCC	American type culture collection
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
NPs	Nanoparticles
OD	Optical density
ROS	Reactive oxygen species
L929	Mouse fibroblast
LB	Luria-bertani
FBS	Fetal bovine serum
P25	Pure titanium dioxide
PBS	Phosphate buffer saline
RPMI	Roswell Park Memorial Institute
$Cu(NO_3)_2 \cdot 3H_2O$	Copper (II) nitrate trihydrate
$Zn(NO_3)_2 \cdot 6H_2O$	Zinc nitrate hexahydrate
ZnO	Zinc oxide
TiO_2	Titanium dioxide
CuO	Copper oxide

Ag	Silver
Au	Gold
$CaCO_3$	Calcium carbonate
Cs	Chitosan
DPPH	2,2-diphenylpicrylhydrazyl
XRD	X-ray powder diffraction
SEM	Scanning electron microscope
TEM	Transmission electron microscope
EDAX	Energy Dispersive Spectroscopy
UV-vis	Ultraviolet–visible spectroscopy
FTIR	Fourier transform infrared spectroscopy
AAS	Atomic absorption spectroscopy
ICP	Inductively coupled plasma
Alg	Alginate
UV	Ultraviolet
Hepes	Hydroxyethyl piperazineethanesulfonic acid
CO_2	Carbon dioxide
SD	*Sprague–Dawley*
ZOI	Zone of inhibition
Ca	Calcium
C	Carbon
Mg	Magnesium
S	Sulphur
K	Potassium
Cl	Chlorine
Al	Aluminium
N	Nitrogen
O	Oxygen
TMTC	Too Many To Count
PUs	Pressure ulcer

LIST OF APPENDICES

PEMBANGUNAN PEMBALUT LUKA ANTIBAKTERIA BERASASKAN KITOSAN BERSAMA NANOPARTIKEL ZNO/CUO MENGGUNAKAN *CALOTROPIS GIGANTEA*

ABSTRAK

Jangkitan kulit yang disebabkan oleh pengkolonian patogen kulit, khususnya yang dikaitkan dengan luka terbuka dan kerintangan antibiotik, merupakan masalah yang besar dalam bidang kesihatan dan perubatan. Pada masa ini, saintis mencari teknologi alternatif bagi balutan luka berasaskan nano yang efisien, khususnya ejen penyembuhan luka transkulit yang bersifat antibakteria bagi menghindari jangkitan luka terbuka. Dalam kajian ini, profil nanokomposit ZnO/CuO yang digabungkan dengan biopolimer berasaskan kitosan (Cs) dikaji, khususnya dari sudut sifat fizikokimia, antibakteria, kesitoserasian dan pemulihan luka. Nanokomposit ZnO/CuO-Cs ini diperoleh melalui proses sejuk beku-pencairan dan dicirikan oleh XRD, SEM, TEM, EDAX, FTIR, AAS, ICP dan spektroskopi UV-vis. Keupayaan antibakteria turut dikaji melalui kaedah resapan cakera Kirby-Bauer, asai masa kematian, dan asai biofilem bagi patogen kulit, manakala keupayaan kesitotoksikan dinilai mengikut garis panduan ISO 10993-5. Pada awalnya, nanopartikel ZnO dan CuO yang berperantara tumbuhan daripada *Calotropis gigantea* (*C. gigantea*) dikenal pasti sebagai ejen antibakteria yang kuat melawan patogen kulit berbanding nanopartikel TiO_2. Seterusnya, nanokomposit ZnO/CuO dedua berkalsin suhu rendah dengan komposisi 75 wt.% ZnO dan 25 wt.% CuO yang dibangunkan menunjukkan aktiviti bakterisid yang dipertingkat dengan nisbah toleransi ≤ 2 dan ≤ 4 bagi semua patogen kulit MDR (pelbagai kerintangan antibiotik) dan bukan MDR yang diuji,

masing-masing. Selanjutnya, nanokomposit ZnO/CuO-Cs berkepekatan rendah (1 wt.%) menunjukkan sifat bakterisid yang berpotensi besar melawan semua patogen kulit yang diuji seperti *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella pneumoniae* (*K. pneumoniae*) dan methicillin-resistant *S. aureus* (MRSA) dengan kebolehhidupan sel yang diperbaik (168.52±14.46 %) pada masa dedahan 72 jam. Tambahan pula, kadar penutupan luka yang pantas (62.35±9.46 %) dan kadar pemindahan sel yang amat baik (26.81 μm/j) dapat dilihat dengan jelas bagi kumpulan yang dirawat dengan ZnO/CuO-Cs-1wt.% melalui asai calar luka *in vitro*. Dengan menggabungkan 1 wt.% nanokomposit ZnO/CuO dedua di dalam biopolimer Cs, ia menghasilkan tahap pH yang berasid, suhu lebur yang lebih tinggi, sifat mekanikal dan sifat hidrofilik yang diperbaik, keliangan tertinggi dan indeks mengembung yang amat baik. Selain itu, kadar degradasi sampel ZnO/CuO-Cs-1wt.% lebih tinggi daripada sampel kawalan komersial apabila ia larut sepenuhnya dalam masa 8 hari. Selanjutnya, kajian haiwan *in vivo* membuktikan bahan balutan luka ini menunjukkan kadar penutupan luka yang amat baik (86.44±6.48 %) dalam masa 14 hari pada tikus *Sprague–Dawley* (*SD*) berbanding Cs dan produk komersial. Kesimpulannya, balutan luka hijau yang dibangunkan ini mempunyai potensi yang amat baik dalam bidang biobahan.

DEVELOPMENT OF ANTIBACTERIAL CHITOSAN-BASED WOUND DRESSING INCORPORATED WITH ZNO/CUO NANOPARTICLES USING *CALOTROPIS GIGANTEA*

ABSTRACT

Skin infections caused by colonization of skin pathogens, especially those associated with open wound and antibiotic resistance, are a significant healthcare problem. Presently, scientists are looking for an efficient alternative nano-based wound dressing technology, particularly antibacterial transdermal wound healing agent in tackling open wound infections. In this work, ZnO/CuO nanocomposites incorporated in chitosan (Cs) based biopolymer were studied for physicochemical, antibacterial, cytocompatibility and wound healing profiles. This ZnO/CuO-Cs nanocomposites were obtained by freeze-thawing process and characterized by XRD, SEM, TEM, EDAX, FTIR, AAS, ICP and UV-vis spectroscopy. Antibacterial potential by Kirby-Bauer disc diffusion method, time kill assay, biofilm assay was studied against skin pathogens and cytotoxicity potential we assessed according to ISO 10993-5 guidelines. Initially, green synthesized ZnO and CuO nanoparticles from *Calotropis gigantea (C. gigantea*) has been identified as a strong antibacterial agent against skin pathogens than TiO_2 nanoparticles. In next, development of low temperature calcined binary ZnO/CuO nanocomposites with compositions of 75 wt.% of ZnO and 25 wt.% of CuO has shown an enhanced bactericidal activity with the tolerance ratio of ≤ 2 and ≤ 4 for all tested MDR (multi drug resistant) and non-MDR skin pathogens, respectively. Subsequently, the low-concentration ZnO/CuO-Cs nanocomposites (1 wt.%) had promising bactericidal property against all

tested skin pathogens such as *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella pneumoniae* (*K. pneumoniae*) and methicillin-resistant *S. aureus* (MRSA) with an improved cell viability (168.52±14.46 %) at 72 hours exposure time. In addition, rapid wound closure rate (62.35±9.46 %) and remarkable cell migration rate (26.81 µm/h) were clearly seen for the ZnO/CuO-Cs-1wt.% treated group through *in vitro* wound scratch assay. Incorporation of 1 wt.% of binary ZnO/CuO nanocomposites in Cs biopolymer created an acidic pH level, higher melting temperature, an improved mechanical properties and hydrophilic nature, highest porosity and remarkable swelling index. Besides, the degradation rate of ZnO/CuO-Cs-1wt.% sample was greater than commercial control sample where it could be fully dissolved in 8 days. Moreover, *in vivo* animal work proved that this wound dressing material has remarkable wound closure rate (86.44±6.48 %) in 14 days toward *Sprague–Dawley* (*SD*) rats than Cs and commercial product. Conclusively, this developed green wound dressing has emerged a promising potential in biomaterials.

CHAPTER 1

INTRODUCTION

1.1 Research background

Bacterial skin infections are one of the main factors affecting wound healing process (Omar et al. 2016). Bacterial colonization on open wound should be reduced to minimum to allow the wound to heal effectively. Generally, wound healing involves several systemic overlapping phases such as hemostatis, inflammation, proliferation and remodelling (Mirhaj et al. 2022; Monika et al. 2022). But these naturally occurring healing process is might be slower and disrupted for diabetic patients.

Prolong not efficient intravenous antibiotics therapies in diabetic patients could lead to high rate of mortality, 85% of major amputation and severe cost of treatment which is nearly 45,000 United States Dollar (N. Amirrah et al. 2020). Importantly, diabetic wounds require serious attention and immediate treatments. As a result of poor blood circulation and reduced tissue oxygenation, the wound healing in diabetic patients is tragically delayed (Spampinato et al. 2020). An antibacterial modern/active dressing is an only way to achieve efficient wound healing for diabetic chronic ulcers.

Antibacterial modern wound dressing material consists of polymer matrix combined with different types of antibacterial agents (Carrow and Gaharwar 2015). Antibacterial agents can be further classified into organic compound such as nano-calcium carbonate (Wang et al. 2017), inorganic compound such as copper oxide (CuO), zinc oxide (ZnO), titanium dioxide (TiO_2), silver (Ag), gold (Au) and copper (Cu) (Mohandas et al. 2018; Bonilla et al. 2019) and natural plant extract (Bairagi et al. 2018).

Generally, the size of antibacterial nanoparticles (NPs) (Elkhawass et al. 2015; Jia et al. 2017; Cho et al. 2018) and concentration of NPs (Sukhanova et al. 2018; Ashajyothi et al. 2018; Mazaheri et al. 2019) plays a vital role in determining the antibacterial and cytocompatibility properties. Anyhow, studies revealed that single and binary NPs incorporated polymers like chitosan (Cs) (Bonilla et al. 2019), polyvinyl alcohol (Kamoun et al. 2015), poly lactic-co glycolic acid (Gupta and Xie 2018), polypyrrole/polyaniline (Maráková a et al. 2017), polypropylene (Delgado et al. 2011; Palza et al. 2015), polyamide (Kumar and Munstedt 2005) and low-density polyethylene (Hu et al. 2018) exhibit enhanced antibacterial and cytocompatibility properties.

Nowadays, the use of nanoparticulated polymer wound dressing has grasped great importance from scientists in tackling bacterial skin infections. Remarkably, medium molecular weight Cs biopolymer that represent a unique characteristic such as non-toxic, biodegradable, antibacterial, biocompatible and wound healing stimulator for development of novel wound dressing material (Shin et al. 2001). Interestingly, visible light active ZnO and CuO NPs exhibited most prominent antibacterial effects towards MDR (multi drug resistant) pathogen (Jan et al. 2019). *Calotropis gigantea* (*C. gigantea*) is a species of traditional medicinal plant from Asclepiadaceae family has been used in treating many types of skin ailments and wound healing application (Misra et al. 2018).

However, some commonly used commercial wound dressing materials are less convincible and exhibit several limitations such as ineffective for self-healing (Zheng et al. 2020), susceptibility to MDR bacterial skin infections (Zheng et al. 2020), lack of biocompatibility to skin cells (Liang et al. 2022), nonbiodegradability (Balas et al. 2021) and poor physicochemical properties (Naomi et al. 2020).

Therefore, in this work antibacterial properties of single NPs calcined at different calcination temperatures (i.e., CuO, ZnO and TiO_2) was investigated against MDR and non-MDR strains. However, it is also identified that these single NPs is capable in performing reasonable bactericidal activity towards certain types of skin pathogens only (Jones et al. 2007; Jan et al. 2018; Gnanam et al. 2019) and the performance of NPs are varied with tunable morphologies such as size, shape and specific surface area (SSA) (Gnanam et al. 2019; Olejnik et al. 2021; Sayed et al. 2022).

Thus, the bactericidal activity of binary NPs calcined at different calcination temperatures and compositions towards MDR and non-MDR skin pathogens was further explored in order to determine most enhanced new formulated synergistic antibacterial binary ZnO/CuO NPs for current wound healing applications. Based on previous study, cytocompatibility of the commercially available wound dressing materials towards human cells were remain unknown (Eberlein et al. 2012; Finnegan & Percival 2015) and the detailed cytocompatibility effects of nanoparticulated wound dressing materials on mouse fibroblast cell line are not well studied. As such, this work focuses on the preparation of ZnO/CuO-Cs nanocomposites and investigates the cytocompatibility effects towards L929 cell line.

According to the previous studies, it was noticed that the inhomogeneous dispersion or distribution, agglomeration/aggregation and high-concentration of NPs throughout polymer matrix causes poor antibacterial efficacy and deterioration of mechanical properties of the wound dressing materials (Thoniyot et al. 2015; Zare 2016; Cho et al. 2021). Commonly, individual NPs tend to agglomerate rapidly as large clusters due to the van der Waals forces (Endres et al. 2021).

Hence in this study, mixing and mechanical stirring under room temperature was performed to obtain homogeneous mixtures of ZnO/CuO-Cs nanocomposites with incorporation of different weight percentage (wt.%) of binary ZnO/CuO NPs. Nowadays, non-biodegradability of commercially available wound dressing materials causes serious environmental land pollution (Abuhamed et al. 2021). This concerned environmental issue can be overcome by introducing ZnO/CuO-Cs nanocomposites which are able to degrade naturally by soil microorganisms. Overall, the present study aimed to fabricate structurally-stable ZnO/CuO-Cs nanocomposites with an improved bactericidal, cytocompatibility and wound healing profiles.

1.2 Problem statement

The main aim of the research work is to have nanoparticulated Cs biopolymer that could heal colonized open wounds by tackling divergent skin pathogens. Currently, biodegradable Cs biopolymer has grasped countless attention for wound healing application. However, this plain Cs biopolymer are less effective towards MDR bacteria and structurally unstable causing an adverse impact on the patients' quality of life. Therefore, transformation of plain Cs biopolymer to antibacterial polymer nanocomposites with embedment of NPs through nanotechnology and green synthesis approach shed light for an efficient wound healing management.

Presently, lack of effectiveness of commercially available wound dressing material is one of the primary factors affecting the wound healing. It was stated that, 75% of wound recovery only achieved at 24 weeks when introduced with Suprasorb X+PHNB (Lohmann and rauscher GmbH) (Eberlein et al. 2012; Finnegan & Percival 2015). Besides, antibiotics are less effective in treating infected open wounds by MDR bacteria.

Isolates of *Pseudomonas aeruginosa* (*P. aeruginosa*) showed 100% of resistance to ampicillin, amoxicillin/clavulanic acid, ertapenem and trimethoprim/sulfamethoxazole (Bessa et al. 2015). Moreover, 21.8% of *Staphylococcus aureus* (*S. aureus*) were resistant to oxacillin (Bessa et al. 2015). Meanwhile, *Corynebacterium* spp. showed full resistance to oxacillin (Bessa et al. 2015).

1.3 Research objectives

1.3.1 General objective

To develop and characterize antibacterial ZnO/CuO-Cs dressing material using green technologies for wound healing application.

1.3.2 Specific objectives

1. To synthesize, optimize and characterize antibacterial agents of ZnO/CuO NPs using *C. gigantea* leaves towards skin pathogens.
2. To evaluate differential weight percentage of binary ZnO/CuO NPs embedded in Cs biopolymer for optimum structural, mechanical, and bactericidal properties against MDR and non-MDR pathogens.
3. To analyze the cytocompatibility and wound healing properties using *in vitro* model.
4. To demonstrate the efficiency of antibacterial polymer nanocomposites using *in vivo* excisional wound healing model.

1.4 Scope of the study

This work was conducted mainly to fabricate ZnO/CuO-Cs nanocomposites with an excellent antibacterial, cytocompatibility and structural properties for wound healing applications. As a result, this work has been divided into 4 phases. Firstly, species of medicinal plant, reducing/stabilizing agents in NPs green synthesis and antioxidant activity of *C. gigantea* leaves extract was characterized via herbarium, HPLC and DPPH assay. Then, single NPs (i.e., CuO, ZnO and TiO_2) and binary ZnO/CuO NPs are synthesized at different calcination temperatures using *C. gigantea* leaves extract and characterized using several material testing lab equipment and antibacterial tests. From this, a strong binary ZnO/CuO NPs produced at low calcination temperature for excellent bactericidal activity was determined.

Subsequently, binary ZnO/CuO NPs are investigated at different compositions and characterized using several material testing lab instruments and antibacterial tests. At the end of this phase, bactericidal properties of binary ZnO/CuO NPs were optimized and best formulation (75 wt.% of ZnO and 25 wt.% of CuO) for excellent antibacterial activity towards all tested skin pathogens was determined. At last, binary ZnO/CuO NPs with different-concentration (1, 3 and 5 wt.%) in Cs biopolymer are investigated and characterized using numerous engineering lab equipment, antibacterial tests, *in vitro* cytocompatibility assay and wound healing assay. From this, the most suitable concentration of binary ZnO/CuO NPs (1 wt.%) in Cs biopolymer was fixed based on cytocompatibility effect and bactericidal efficacy of sample. Besides, an improved wound closure and cell migration rate for optimized ZnO/CuO-Cs nanocomposite was successfully determined.

1.5 Outcomes

At the initial stage of this work, the excellent combination of binary ZnO/CuO NPs was fabricated under low calcination temperature for an improved bactericidal performance. Subsequently, the excellent composition of binary ZnO/CuO NPs for an improved antibacterial activity was determined. Thereafter, the optimized low-concentration ZnO/CuO-Cs nanocomposites with an excellent antibacterial, cytocompatibility and structural properties for wound healing applications was successfully characterized. Eventually, the remarkable wound closure and cell migration rate for low-concentration ZnO/CuO-Cs nanocomposites were captured through *in vitro* and *in vivo* studies.

CHAPTER 2

LITERATURE REVIEW

2.1 Physiology of wound healing

Present wound dressing materials have limitation in treating skin pathogens colonization that is associated with open wound infections. Recovery phases of chronic open wound ulcers remains a great challenge in wound management care due to the influence of several local and systemic factors. Presently, polymer-based nanocomposites incorporated green synthesized bactericidal agents have received countless attention and shown multiple benefits to tackle the mention limitation.

Wound means an interference to the physiological arrangement of the skin cells and a disturbance to its function in protecting underlying tissues and organs (Omar et al. 2016). Wound can be further categorized into two types based on the healing recovery time which are acute wound and chronic wound. Acute wound can recover within a limited amount of time without any further medical complication while chronic wound shown no sign of healing within 8-12 weeks of time (Omar et al. 2016).

Wound healing is a biological process which involves four overlapping healing stages such as hemostasis, inflammation, proliferation and remodelling (Figure 2.1). In this hemostatis phase, platelets knot is activated instantly right after the tissue injury and rupture of blood vessels (Spampinato et al. 2020; Nguyen et al. 2023). The excessive blood lost is greatly reduced and formation of clot or thrombus is permanently sealed with presence of activated platelets.

The inflammatory or swelling phase is further improved with presence of several vital growth factors such as epithelial growth factor (EGF), vascular endothelial growth factor (VEGF) and cytokines (Spampinato et al. 2020; Nguyen et al. 2023). These growth factors primarily responsible in generating neutrophils/white blood cells, monocytes and lymphocytes. It eliminates debris of cells, foreign bodies and colonization of skin pathogens from the targeted wound site. The granulated tissue and fibroblast proliferation are effectively enhanced with migration of keratinocytes.

In the proliferation phase the dermis is restored with new blood vessels when the cytokines play an important role in accelerating wound re-epithelization, angiogenesis, collagen and elastin synthesis (Spampinato et al. 2020; Nguyen et al. 2023). During the final remodelling session, most of the newly produced capillaries undergo regression along with apoptosis of macrophages and fibroblast. The wound healing phenomenon is controlled by numerous intrinsic factors and extrinsic factors. Intrinsic factors comprise drugs, diseases, rheumatoid arthritis, acute shock, ageing, free radicals, oedema and oxygen, while extrinsic factors include pressure, wound temperature, exudates and application of wound dressing material (Singh et al. 2013).

An open wound is a type of external skin injury which results from abrasion, laceration, avulsion, puncture, burn and surgical incision. Common type of non-infected open wounds is treatable with several medical treatments using antibiotics ointment or cream, natural remedies from medicinal plant, and commercially available antibacterial wound dressing materials. But locally infected open wound injuries could lead to human morbidity and mortality, including amputation and death (Nussbaum et al. 2018).

Contaminated open wound arising from the colonization and growth of Gram-positive bacteria, Gram-negative bacteria, and MDR bacteria are serious health-care issue that gravely affect human skin. Non-MDR Gram-positive *S. aureus* is also one of the most common skin pathogens responsible for infectious open wound ulcers (Wong et al. 2015; Du et al. 2022; Said et al. 2022). Study showed that, approximately 37% of hospitalized patients with surgical wound are contaminated with *S. aureus* strain (Chambers and DeLeo 2009; Bessa et al. 2015; Ventola 2015).

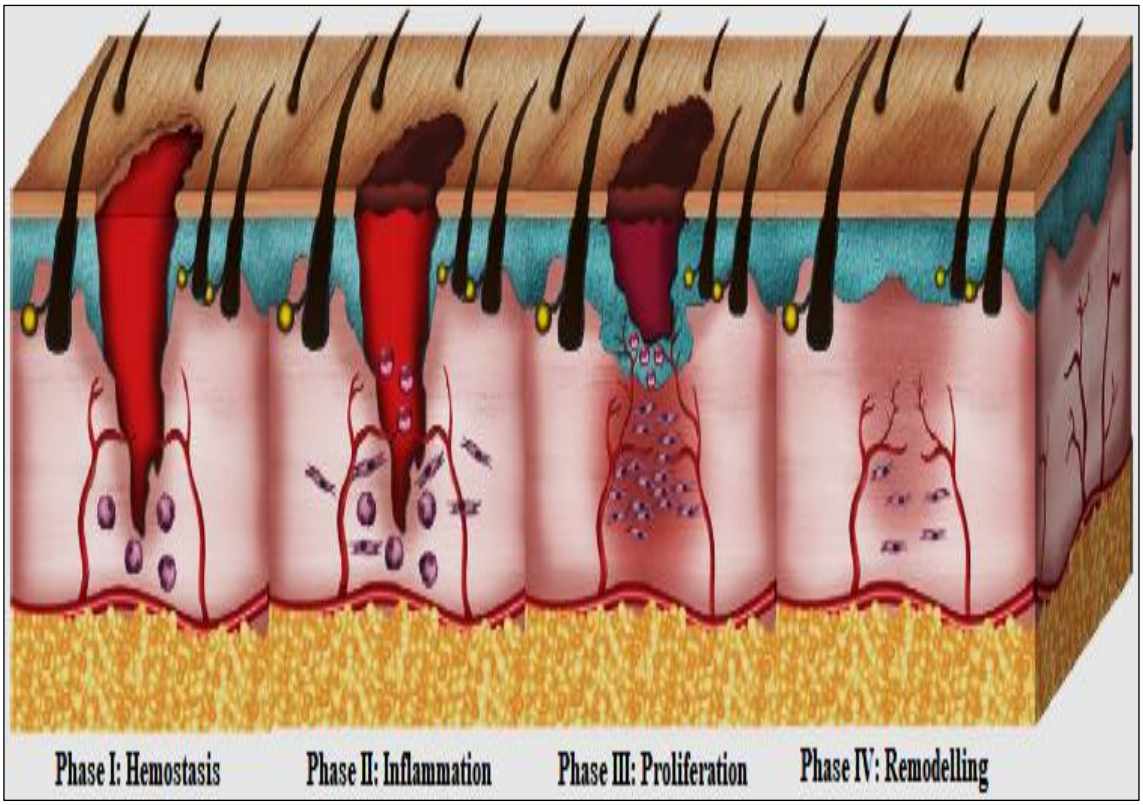

Figure 2.1 **Four phases of wound healing.** Predictable phases of hemostasis, inflammation, proliferation and remodelling. (Adapted from Mirhaj et al. 2022; Monika et al. 2022 used under CC BY-NC-ND 4.0)

Skin pressure ulcers (PUs) are colonized open wounds that develop on the skin as a result of pressure or friction on one spot of the patient's body. Several pathogens, such as *S. aureus* (37%), *P. aeruginosa* (17%), *Proteus mirabilis* (*P. mirabilis*) (10%), *Escherichia coli* (*E. coli*) (6%) and *Corynebacterium* spp. (5%), have been widely studied and associated with ulcerative skin disease (Bessa et al. 2015; Braga et al. 2016; Omar et al. 2016) (Figure 2.2).

A detailed quantitative study reported that the prevalence rates of PUs are 15.5% in Kuala Lumpur, Malaysia (2013) (Khor et al. 2014), 33% in Palestine (2017) (Qaddumi and Almahmoud 2019), and 16% in Bandung, Indonesia (2017) (Sari et al. 2019). Open wound infection has been found in 60 (74.0%) of the collected samples from PUs patients, and these PUs primarily comprise *Enterobacteriaceae* strains (49.0%), such as *E. coli*, *Klebsiella pneumoniae* (*K. pneumoniae*), *Enterobacter* spp., and *Proteus* spp.; followed by *S. aureus* (28.0%) and nonfermenting Gram-negative bacteria (23.0%), mostly *P. aeruginosa*, *Acinetobacter* spp., and methicillin-resistant *S. aureus* (MRSA) (El-Toraei et al. 1977; Park-Lee and Caffrey 2009; Dana and Bauman et al. 2014; Braga et al. 2016).

In the past researchers found that, amongst 77 wound swab samples studied at Local Hospital, Malaysia, 82 isolates consist of Gram-negative (71.1%) and Gram-positive bacteria (27.7%) (Nur Hilda Hanina et al. 2015). According to Yong et al. (2021), *K. pneumoniae* (36.2%), *P. aeruginosa* (26%), *S. aureus* (23.6%), *Streptococcus spp*. (8.7%) and *Acinetobacter baumannii* (*A. baumannii*) (5.5%) were common strains which were isolated from postoperative patients at National Heart Institute of Malaysia at Kuala Lumpur. Therefore, appropriate systemic wound therapy and treatments should be introduced to cure open wound ulcers and tackling bacterial contamination.

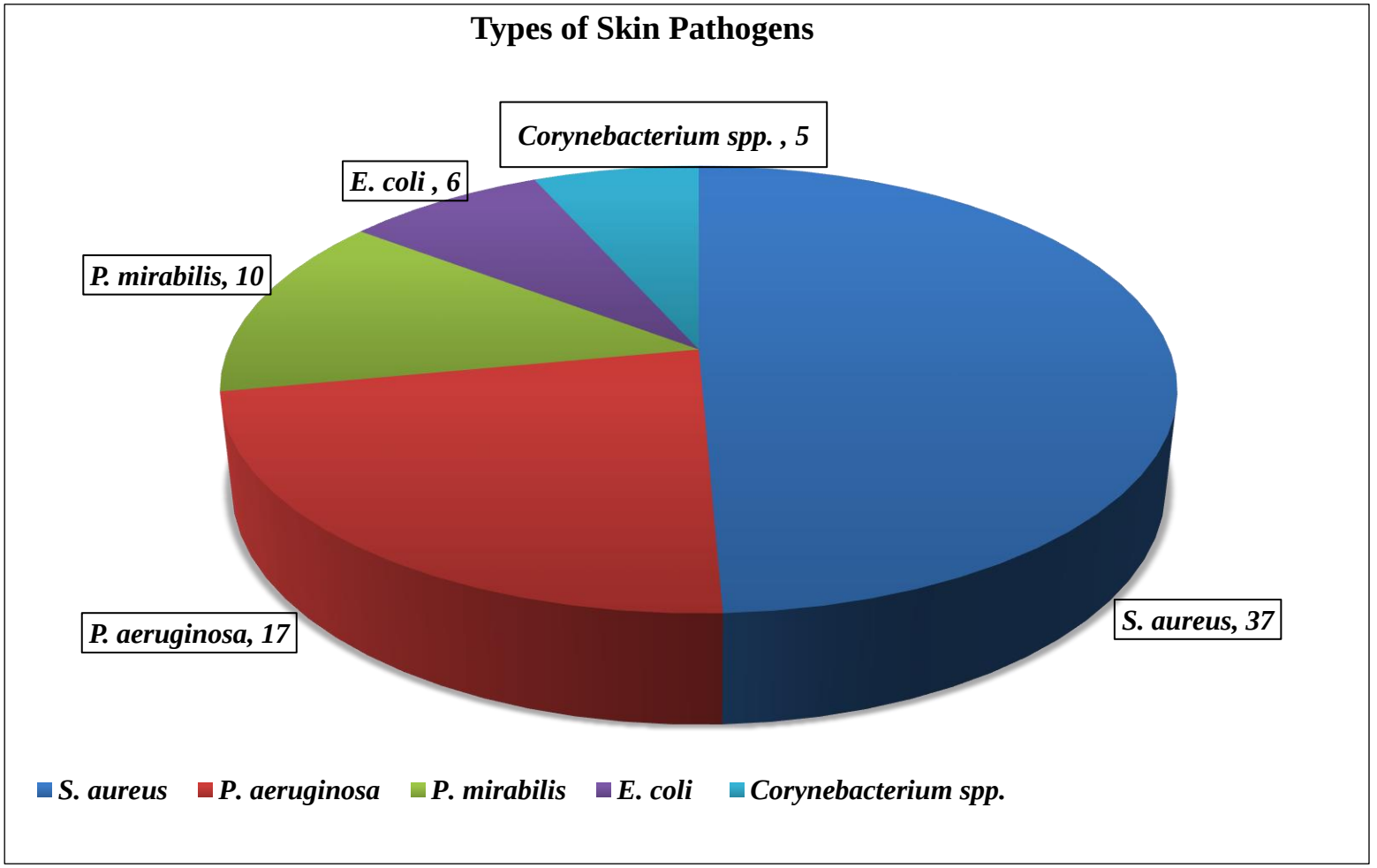

Figure 2.2 **Percentages of common skin pathogens isolated from ulcerative skin disease.** *S. aureus* and *P. aeruginosa* were most causative strains. (Adapted from Bessa et al. 2015).

2.1.1 Types of open wounds

Colonized open wounds are a main cause of mortality and morbidity amongst postoperative patients (Gupta et al. 2015). Types of open wound ulcers include surgical ulcers, diabetic wound, pressure ulcers, arterial ulcers, chronic ulcers, skin disorders and infections, traumatic wounds, venous ulcers, vasculitis ulcer, burns, neuropathic ulcer, and malignant ulcer (Singh et al. 2013; Nussbaum et al. 2018) (Figure 2.3). These types of open wounds are associated with exudate and intense pain and hardly treatable with antibiotics when contaminated by MDR skin pathogens (Duarte et al. 2018).

Diabetic foot ulcer (DFU) patients have high risk of foot amputation and organ damages (Rania et al. 2020). Previous studies have shown that diabetic ulcers cover a financial spending approximately $1.38 billion per year for wound healing care (Urso et al. 2021). In contrast of, elderly long-term bedridden, chair bound or immobile patients with lack of body movement are mostly exposed to pressure ulcers where it could destroy the patient's quality of life (Baumgarten et al. 2009).

Arterial ulcers occurred due to the microvascular pathology and a conservative therapy is needed for the further treatment (Sanchez and Partsch, 2017). Traumatic ulcers on the tongue, lips, and oral mucosa are caused by several reasons such as thermal burn, electrical burn, chemical burn and mechanical damage (Mortazavi et al. 2016). Venous leg ulcers or venous stasis ulcers are also categorized as chronic wound ulcer and mostly seen on the legs and lower extremities of aged patients (Xie et al. 2018; Bernatchez et al. 2022). Normally, neuropathic ulcerative patients have medical history of infected peripheral arterial disease and around 15% of them were facing limb amputation due to untreatable infections (Urso et al. 2021).

Vasculitis is a type of skin ulcers were commonly seen on the foot and lower legs with several inflammatory disorders (Morita et al. 2020). Anti-inflammatory and immunosuppressive drugs medication are used to cure and enhance cell proliferation of vasculitis ulcers (Papi and Papi, 2016). Burns are categorized based on causative agent such as physical thermal burns (i. e., flame and scalds), contact burns, electrical burns (i. e., high or low voltage power), flash burns, laser burns, radiation burns, chemical burns (i. e., acid and alkaline) (Tiwari, 2012). Wound healing and recovery for burns are classified according to the depth of burns (i. e., first-degree burn, second-degree burn and third-degree burn) (Tiwari, 2012).

Malignant leg ulcers are cancerous and require special care and treatment such as proper excision and skin grafting (Hayes and Dodds, 2003). But large malignant ulcers only can be removed through amputation. Nowadays, commercially available wound dressing biomaterials such as gauze, bandages, films, hydrogels, foams, sponges, hydrofibers, tissue engineered skin substitutes, hydrocolloid, alginate and nano Ag are used to accelerate wound healing in longstanding open ulcers which is contaminated by skin pathogens (Kumar et al. 2012; Elbadawy et al. 2015; Bhattacharya and Mishra, 2015; Lu et al. 2017; Han and Ceilley, 2017; Arshad et al. 2019; Nguyen et al. 2019).

Discovering appropriate wound healing therapies to facilitate optimal wound healing and inhibit the skin pathogen growth on open wound ulcers is urgently needed. Nanotechnology in nanoparticulated polymer wound dressing material with antibacterial properties is a most promising strategy in stimulating wound proliferation and angiogenesis. Therefore, a detailed assessment on current wound care management is discussed thoroughly in the next sections.

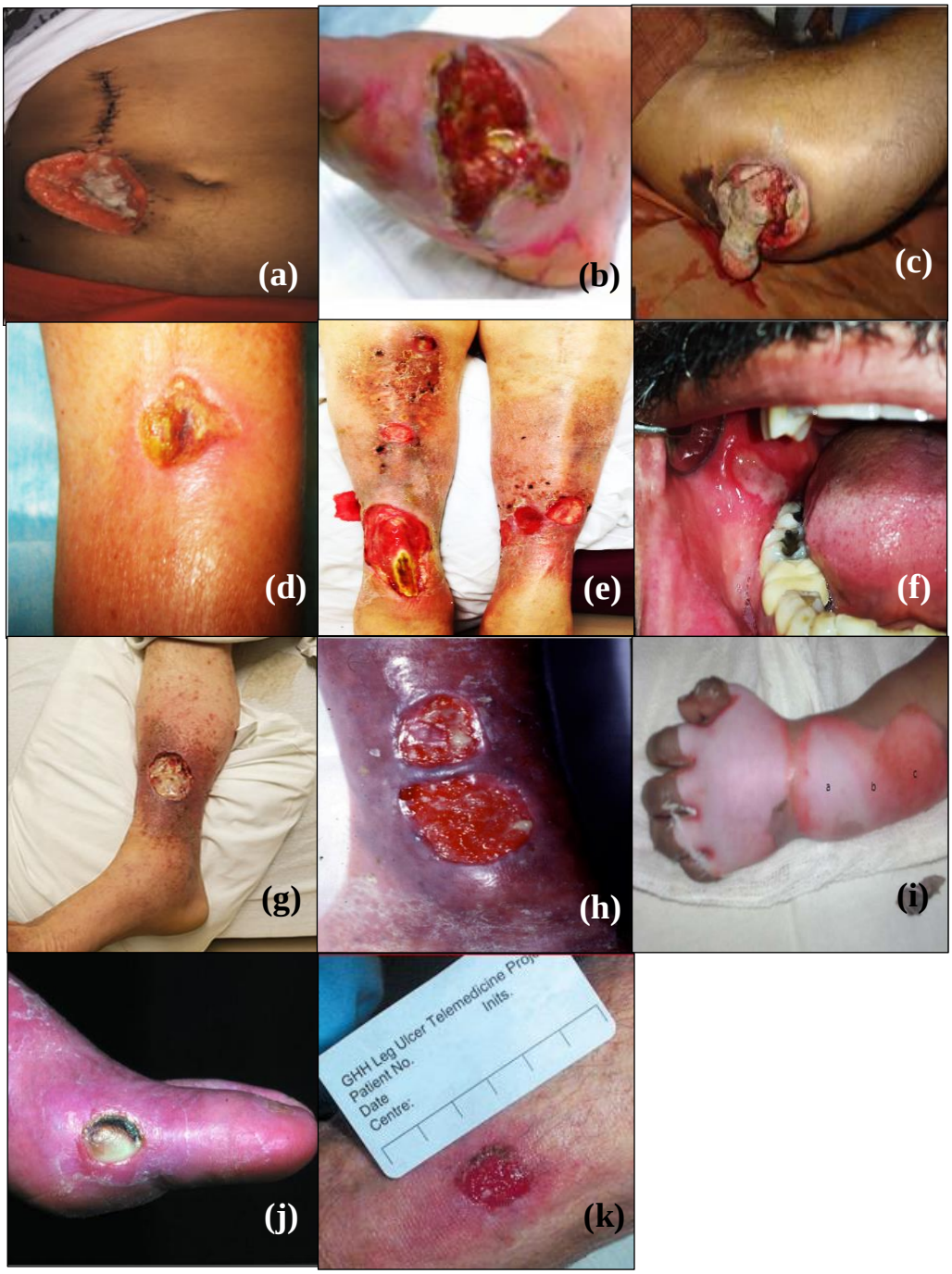

Figure 2.3 **Types of open wound ulcers.** (a) surgical, (b) diabetic, (c) pressure, (d) arterial, (e) chronic, (f) traumatic, (g) venous, (h) vasculitis, (i) burns, (j) neuropathic, and (k) malignant. (Adapted from Duarte et al. 2018; Rania et al. 2020; Bhattacharya et al. 2015; Sanchez and Partsch, 2017; Han and Ceilley, 2017; Mortazavi et al. 2016; Bernatchez et al. 2022; Papi and Papi, 2016; Tiwari, 2012; Urso et al. 2021; Hayes and Dodds, 2003)

2.1.2 Wound care, treatments and managements

This section presents a discussion on current wound care treatments and management. Collagen-based wound dressing biomaterials with encapsulation of antibacterial agent are excellent tissue stimulator and inhibitor of bacterial infection particularly on diabetic foot ulcers recovery by promoting keratinocyte and fibroblast migration (N Amirrah et al. 2020). This type of collagen-based skin substitute shown less toxicity and immunogenic effect and enhance cellular interaction in open wound ulcers. The collagen can be produced from marine, porcine, equine sources, bovine and ovine.

Proper establishment of clinical centres, knowledge and education in wound healing, relevant standards, clinical funding, and transparent politics are most strategic steps for successful wound care management (Mahmoudi and Gould, 2020). There are several wound healing approaches such as skin transplantation, stem cell or cell therapy, instrumental methodology, wound dressing material, pharmacotherapy (i. e., analgesia and antibiotics), revascularization and rehabilitation used in current biomedical field for treating open ulcers (Tharumaraja et al. 2021; Mirhaj et al. 2022).

In general, in tissue grafts, autograft (own tissue cell), allograft (donor tissue cell) or xenograft (animal tissue cell) is considered to treat and replace the damaged skin (Mirhaj et al. 2022). Currently commercially available skin substitutes such as AlloPatch®, AlloPatch® Pliable, AlloSkin™ RT, Coll-e-derm™, Dermapure®, DermaSpan™, FlowerDerm™, GammaGraft™, GraftJacket™, hMatrix® ADM, InteguPly®, Matrix HD®, Biobrane, Transcyte, Apligraf, Dermagraft, Alloderm and Integra are widely used to tackle the affected skin area (Snyder et al. 2020; Mirhaj et al. 2022).

Additionally, stem cells technology which is extracted from adult tissue or embryonic are being introduced in treating non-healing chronic wounds (Mirhaj et al. 2022). Platelets derived from bone marrow megakaryocytes plays a vital role in phases of wound healing. Instrumental methodology includes low-level laser therapy, negative pressure wound therapy, cold atmospheric pressure plasma, and hyperbaric oxygen therapy (Tharumaraja et al. 2021; Mirhaj et al. 2022).

Wound dressings are categorized into three types such as traditional dressing, passive dressing and modern/active dressing (Mirhaj et al. 2022). But nowadays, traditional dressing (i. e., plant, honey, naturopathy and Ayurveda) and passive dressing (i. e., gauze, cotton pads and bandages) have not much attention owing to the lack of physical barrier between wound surface and outer environment, ineffective for self-healing, susceptibility to bacterial infections and low absorptive capacity (Monica et al. 2022; Mirhaj et al. 2022). However, employing a modern/active wound dressing in the form of foams, films, hydrocolloids, and hydrogels will consider most reliable and promising approach in treating colonized open wound ulcers (Figure 2.4).

This modern/active dressing are designed to have strong antibacterial effect, rapid wound re-epithelialization and haemostasis, less painful, maintain proper moisture and pH level, favourably flexible with improved mechanical properties, enhanced cell adhesions, removal of excessive exudate, high permeability of water and gases, biodegradable, biocompatible, swollen, non-toxic, hydrophilic and granular tissue stimulator (Mirhaj et al. 2022). Presently, biopolymer based wound dressings such as chitin, chitosan, sodium alginate, calcium alginate, fibrinogen, and collagen I are widely used for treating open ulcers (Tharumaraja et al. 2021; Mirhaj et al. 2022).

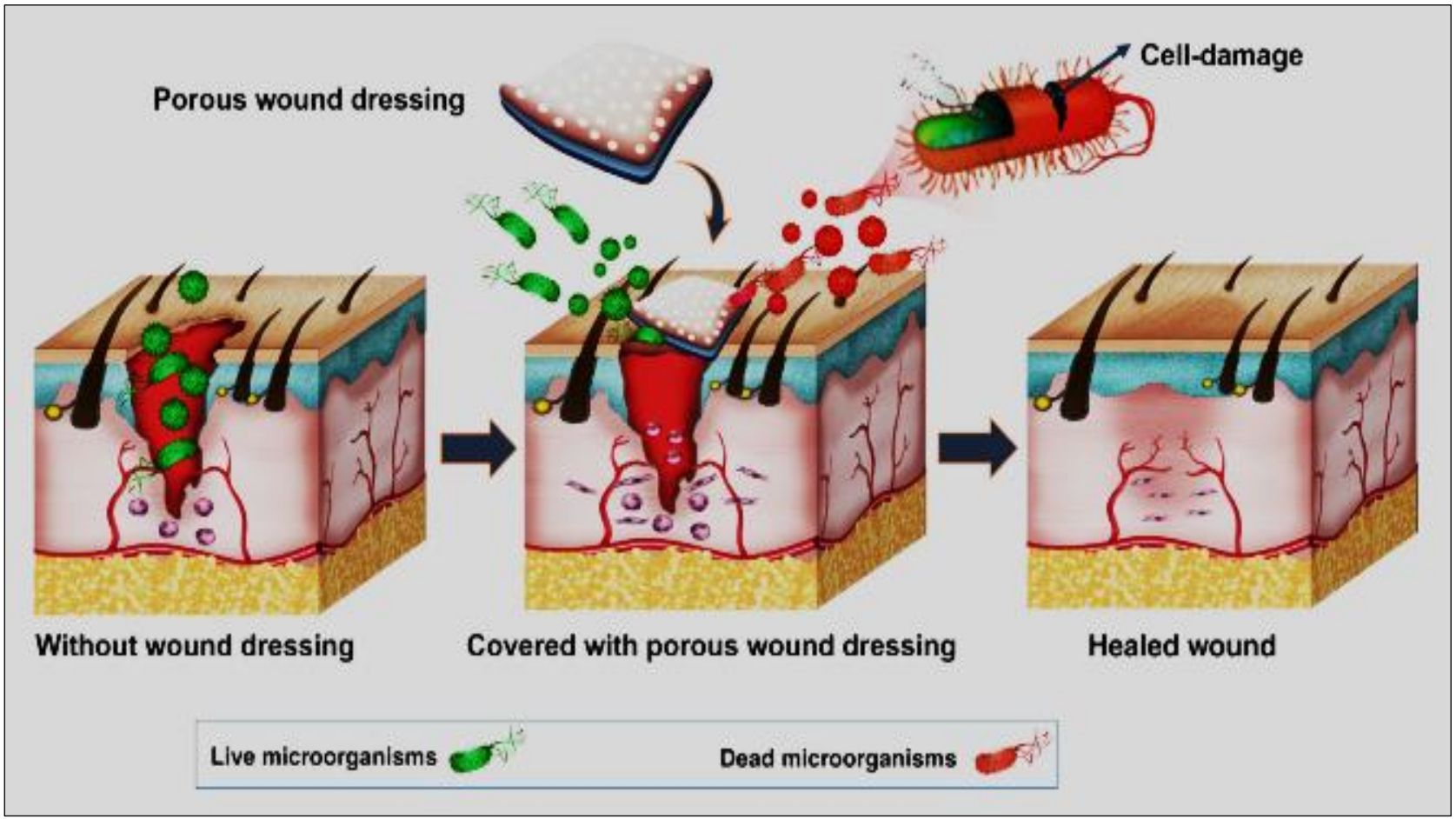

Figure 2.4 **An ideal antibacterial and biocompatible wound dressing material.** The open wound healed completely after administration of antibacterial porous wound dressing material. (Reproduced from Mirhaj et al. 2022 is licensed under CC BY-NC-ND 4.0)

2.1.3 Limitations and challenges

Currently, chronic surgical and diabetic wound healing are greatest challenge and considerable burden in health care and wound management system. Wounds infected by MDR and non-MDR skin pathogens are hardly treatable (Uckay et al. 2015; September et al. 2019; Wang et al. 2019). Medicare cost estimates for the treatment of all wound types range from $28.1 billion to $96.8 billion, and the greatest expenses are for surgical ulcers, followed by diabetic wounds (Nussbaum et al. 2018).

Broad-spectrum antibiotics, such as vancomycin, oxacillin, penicillin, cefoxitin and chloramphenicol, are ineffective to control the growth of MDR skin pathogens and are not preferred in colonized open wound ulcers (Hamzah et al. 2019). Besides that, prolong not efficient intravenous antibiotic therapies could lead to tortuous hospitalization procedures and amputation (Paydar et al. 2006). Moreover, a recent study showed that roughly 23,000 deaths a year in the United States and more than 33,000 deaths in Europe are due to antibiotics failure in treating MDR strain-infected wound ulcers (Pacios et al. 2020).

2.2 Potential of *Calotropis gigantea*

The *C. gigantea* (*L.*) *Dryand*. (*C. gigantea*) plant from Asclepiadaceae family has attracted attention in terms of wound healing (Radhakrishnan et al. 2015a, b; Ayodhya and Veerabhadram 2017) and treatment of infected skin ulcers (Kumar et al. 2010; Patil and Saini 2012). The mechanisms underlying the antibacterial activity of the *C. gigantea* are due to the phytochemicals (i. e., phenolics, flavonoids, terpenes, etc.) (Kumar et al. 2010). These phytochemical compounds act as reducing and capping agents in the green synthesis of NPs (Rajkuberan et al. 2015; Sharma et al. 2015).

2.2.1 Plant description

C. gigantea is a well-known medicinal plant in treating several skin diseases in past years (Figure 2.5). It is also known as “Erukku”, “Giant Milkweed”, “Sweta Arka”, “Swallow Wort”, “Boro Akanda” and “Crown Flower” (Habib and Karim, 2011; Negi and Bisht, 2021). It is also called as Urkkovi, Manakkovi, Mannakovi and Errukalai in Tamil (Abeysinghe, 2018). It is well recognized as Remiga, Rembega and Kemengu in Malaysia (Pandian et al. 2013; Negi and Bisht, 2021).

In ancient Greek, *Calotropis* means “beautiful boat keel” (Al Sulaibi et al. 2020). The milky and evergreen flowering plant of *C. gigantea* is a genus from family of Asclepiadaceae. It grows wildly in region of Africa and South East Asia such as Malaysia, Thailand, Cambodia, Burma, China, India, Indonesia, Pakistan, Philippines, Sri Lanka, Bangladesh and in the sub-Himalayan tract (Habib and Karim, 2011; Abeysinghe, 2018; Bhardwaj and Misra, 2018). This species can be found in the sandy, abandoned, and well-drained soil land at the elevations of 1000 m (Negi and Bisht, 2021).

This fast growing and evergreen flowering plant with 4-5m tall, could withstand against strong wind, soil salinity and drought (Negi and Bisht, 2021). It has large and light green leaves with elliptic-ovate shape with size of 10-20 cm long and 8-10 cm wide. It has poisonous milky stem which contain of latex. The long-lasting waxy crown flower growth in clusters, pale white or pale purple in color with a profusion of equally five-petaled (Figure 2.6a). The single or paired fruits are plump, swelled and saccate, to 6×3 cm, ovoid (Figure 2.6b). The seeds are compacted in silky white pappus (Figure 2.6c). The feathery pappus seeds can be distributed by water and wind.

Figure 2.5 **The *C. gigantea* plant.** It's also called as "*Erukku"*, "giant milkweed" and "crown flower".

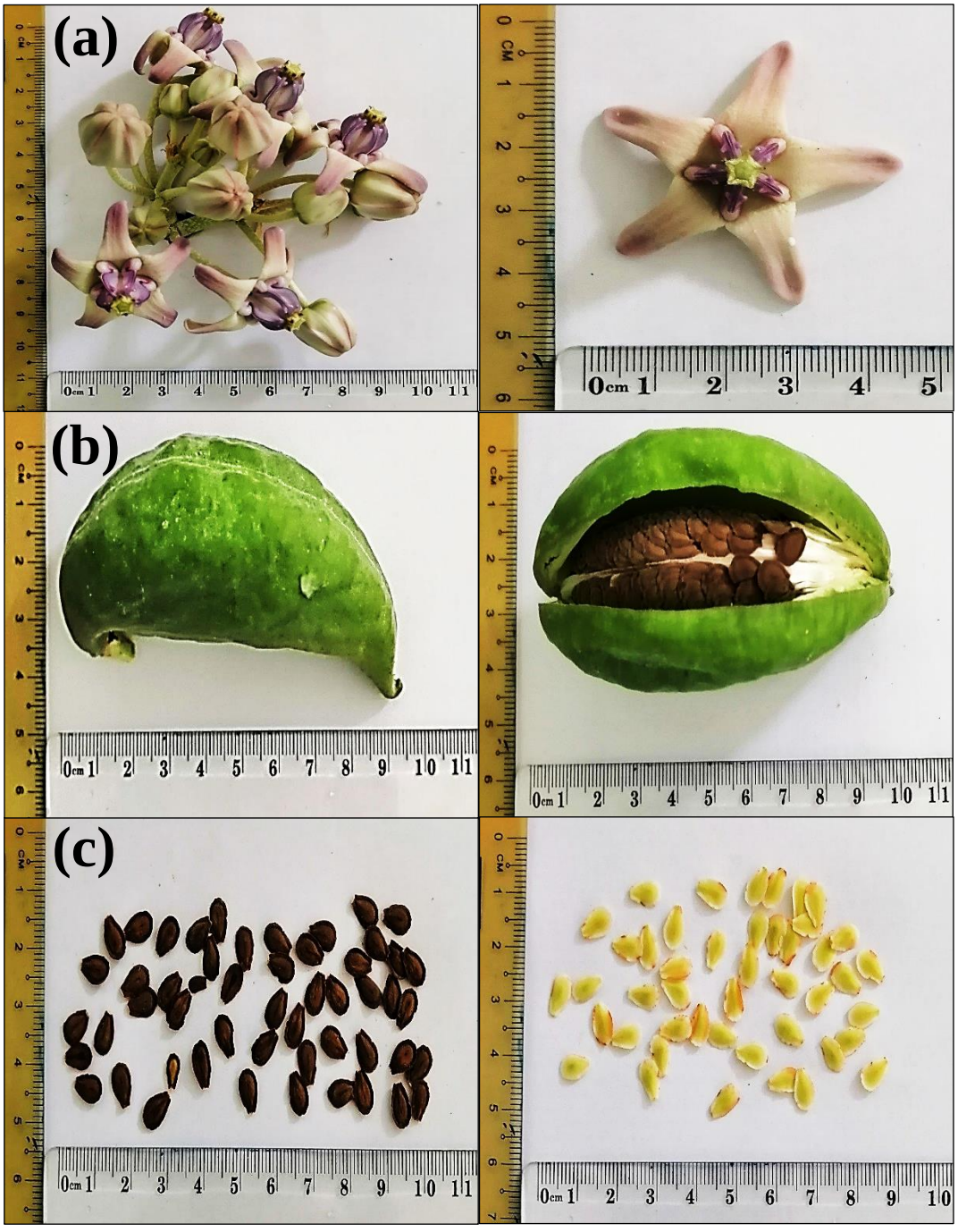

Figure 2.6 **The flowers, fruits and seeds of the *C. gigantea* plant.** (a) Five petals crown flowers, (b) fruits and (c) seeds.

2.2.2 Traditional application

This section presents a discussion on the traditional application of *C. gigantea.* This medicinal plant with microbicidal properties is often used to treat various skin infections (Kumar et al. 2010) and skin ulcers (Sangeetha et al. 2020). The traditional use of this plant in treating open ulcers is well documented (Sharma et al. 2015). Phytochemical agents such as flavonoids and terpenoids have shown excellent antimicrobial and wound healing properties (Alafnan et al. 2021). It is quite popular amongst Indian practitioners in Konar community of Tamil Nadu in Ayurvedic medicine.

It is commonly reserved for treating skin, digestive, neurological disorders, fever, bacterial infection, vomiting, respiratory, circulatory, asthma, colds, coughs, diarrhoea, indigestion, leprosy, leukoderma, ailments of spleen and liver, toothache, caries, ear ache, mental and dental disorders, stings, sprain, ringworm, syphilis, anxiety, pain, tuberculous, epilepsy, skin diseases, boils and sores, ulcers, piles, malaria and rheumatism ailments (Bhardwaj and Misra, 2018; Jayakumar et al. 2018; Abeysinghe, 2018). It has been used as analgesic, procoagulant and wound healing agent as well (Aderounmu et al. 2013; Abeysinghe, 2018).

The convincing antimicrobial, antifungal, anti-candida, anticancer, antioxidant, analgesic activity, insecticidal activity, anti-keloidal, cytotoxic, antipyretic, anti-asthmatic, anti-inflammatory, wound healing, anti-diarrheal, anxiolytic, sedative, hepatoprotective, antidiabetic, larvicidal and enzyme inhibitory properties of *C. gigantea* were also well proven (Habib and Karim, 2011; Aderounmu et al. 2013; Abeysinghe, 2018; Alafnan et al. 2021; Negi and Bisht, 2021).

2.2.3 Future prospects

Several studies have discovered that inorganic metal oxide antibacterial agents synthesized from this medicinal plant can be amazing alternatives for infectious open wound treatments of PUs because they are abundant in numerous varieties of metal oxides that release antibacterial ions and reactive oxygen species (ROS) which cause increased cell membrane rupture and death in skin pathogens (Sumbal et al. 2019; Alavi and Karim 2018).

The isolation of phytocompounds from *C. gigantea* act as potential inhibitor of COVID-19 Mpro and open doors to development of new drugs (Dutta et al. 2021). In addition, methanolic and chloroform treated root bark of *C. gigantea* and anhydrosophoradiol-3-acetate (A3A) compound which obtained from the *C. gigantea* flower have exhibited *in vitro* antitumor activity (Habib and Karim, 2011; Habib and Karim, 2013). Moreover, several new isolated phytocompounds such as 9′-methoxypinoresinol, calofurfuralside A and calofurfuralside B from leaves of *C. gigantea* showed significant cytotoxicity against human pancreatic cancer cell line (PANC-1) (Nguyen et al. 2017).

On top of that, this medicinal plant has also shed light on the usage in non-biomedical field. Currently, it was fully utilized in multipurpose non-biomedical application such as foam-like oil sorbent, fuel, animal feed, pesticidal, building material, fodder, timber and fibre production, phytoremediation, food, textile, paper industries and insecticidal (Al Sulaibi et al. 2020; Xiao et al. 2021; Kaur et al. 2021).