ACUTE DERMAL TOXICOLOGICAL STUDY OF CHITOSAN/POLYVINYL ALCOHOL/SODIUM ALGINATE COMPOSITE FILM LOADED WITH *Gynura procumbens* EXTRACT AS POTENTIAL BIOFILM PLASTER FOR WOUND HEALING

By

BRADY JOSHUA A/L KODIMANI

A RESEARCH PAPER SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF VETERINARY MEDICINE

FACULTY OF VETERINARY MEDICINE

UNIVERSITI MALAYSIA KELANTAN

2024

ORIGINAL LITERARY WORK DECLARATION

I hereby certify that the work embodied in this thesis is the result of the original research and has not been submitted for a higher degree to any other University or Institution.

☒ **OPEN ACCESS** I agree that my thesis is to be made immediately available as hardcopy or online open access (full text).

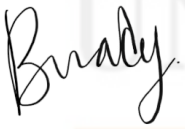
☒ **EMBARGOES** I agree that my thesis is to be made available as hardcopy or online (full text) for a period approved by the Post Graduate Committee.
Dated from_____until_____.

☒ **CONFIDENTAL** (Contains confidential information under the Official Secret Act 1972)*

☒ **RESTRICTED** (Contains restricted information as specified by the organisation where research was done)*

I acknowledge that Universiti Malaysia Kelantan reserves the right as follows.

1. The thesis is the property of Universiti Malaysia Kelantan
2. The library of Universiti Malaysia Kelantan has the right to make copies for the purpose of research only.
3. The library has the right to make copies of the thesis for academic exchange.



SIGNATURE OF CANDIDATE SIGNATURE OF SUPERVISOR

NRIC/PASSPORT NO. 981209086767 ASC. PROF. DR. RUMAIZI BIN SHAARI
DATE: 13/2/2025 DATE: 13/2/2025

Note: * If the thesis is CONFIDENTIAL OR RESTRICTED, please attach the letter from the organization stating the period and reasons for confidentiality and restriction.

ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement of the course DVT 55204 – Research Project.

This research sought to study the effects of novel biofilm containing *Gynura procumbens*, sodium alginate, chitosan, and PVA as prospective for wound dressings. The three different biofilms were constructed using solvent casting method and Soxhlet extraction method. *Gynura procumbens* extracts were incorporated in every biofilm. Each biofilm characterization includes the investigation of Acute Dermal Toxicity, Hematological analysis and histopathology. All rats were observed for any alterations in their physical characteristics and behavioural patterns that might arise as a result of the plant's toxic effects during the 14-day study period. Overall, the findings show that all three biofilms with *Gynura procumbens* extract equipped with chitosan, PVA, and sodium alginate-based films have promising potential as plasters for wound treatment in the future. The minimal changes that the biofilm caused indicates a new plausible plaster to be refined. The study emphasizes on the important aspects of appropriate film synthesis and presence of dermal and systemic toxicity that could pave a path for future development of wound healing plasters. Setting a foundation for further investigation and improvement for biofilms enhanced with *Gynura procumbens* for therapeutic applications, this research advances a potential for a new novel pharmaceutical product.

Keywords: Acute Dermal Toxicity, Polymer Based biofilm, *Gynura procumbens*, Wound healing plaster.

ABSTRAK

Abstrak kertas penyelidikan yang dibentangkan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan, dalam keperluan sebahagian daripada kursus DVT 55204 – Projek Penyelidikan.

Penyelidikan ini bertujuan untuk mengkaji kesan biofilm novel yang mengandungi *Gynura procumbens*, natrium alginat, chitosan, dan PVA sebagai prospek pembalut luka. Tiga biofilem berbeza telah dibina menggunakan kaedah *solvent casting* dan kaedah pengekstrakan Soxhlet. Ekstrak *Gynura procumbens* telah dicampurkan dalam setiap biofilm. Setiap pencirian biofilm termasuk penyiasatan Acute Dermal Toxicity, analisis Hematologi dan histopatologi. Semua tikus diperhatikan untuk sebarang perubahan dalam ciri fizikal dan corak tingkah laku mereka yang mungkin timbul akibat kesan toksik tumbuhan semasa tempoh kajian selama 14 hari. Secara keseluruhan, penemuan menunjukkan bahawa ketiga-tiga biofilem dengan ekstrak Gynura procumbens yang dilengkapi dengan filem berasaskan chitosan, PVA, dan natrium alginat mempunyai potensi yang menjanjikan sebagai plaster untuk rawatan luka pada masa hadapan. Perubahan minimum yang disebabkan oleh biofilm menunjukkan plaster baru yang munasabah untuk diproses. Kajian ini menekankan aspek penting dalam sintesis filem yang sesuai dan kehadiran toksik dermal dan sistemik yang boleh membuka laluan untuk pembangunan masa depan plaster penyembuhan luka. Menetapkan asas untuk penyiasatan lanjut dan penambahbaikan untuk biofilm yang dipertingkatkan dengan *Gynura procumbens* untuk aplikasi terapeutik, penyelidikan ini memajukan potensi untuk produk farmaseutikal baharu di pasaran.

Kata kunci: Acute Dermal Toxicity, biofilm polimer, *Gynura procumbens*, Plaster penyembuhan luka.

CERTIFICATION

This is to certify that we have read this research paper entitled **'ACUTE DERMAL TOXICOLOGICAL STUDY OF CHITOSAN/POLYVINYL ALCOHOL/SODIUM ALGINATE COMPOSITE FILM LOADED WITH *Gynura procumbens* EXTRACT AS POTENTIAL BIOFILM PLASTER FOR WOUND HEALING'** by **Brady Joshua A/L Kodimani**, and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirements for the course DVT 55204 – Research Project.

Associate Professor Dr. Rumaizi Bin Shaari

DVM (UPM), PhD in Veterinary Surgery (University of Adelaide, Australia)

Associate Professor

Faculty of Veterinary Medicine

Universiti Malaysia Kelantan

(Supervisor)

Ts. Dr. Muhammad Luqman Bin Nordin

Senior Lecturer

DVM (UMK), PhD in Pharmacy (UKM)

Universiti Malaya

(Co-supervisor)

ACKNOWLEDGEMENTS

Heartfelt gratitude to those who have given their support, guidance, advice, and financial aid for the completion of this project paper:

TS. DR. MUHAMMAD LUQMAN BIN NORDIN

ASSOCIATE PROFESSOR DR. RUMAIZI BIN SHAARI

DR. FATHIN FAAHIMAAH BINTI ABDUL HAMID

DR. ROSLINA BINTI RASHID

DR. JESSIE CHONG TZE MEI

DR. JESSAMINE THONG JIAH KHER

DR. MUHAMMAD FAIZ BIN MOHD. FIKRI

EII TZE MIN

ROTATION GROUP 2 DVM5

HAZIQ FAHMI BIN MOHD DALI

BATRISYA BAZLA BINTI HUSAIN

Lab assistants of FPV UMK

DVM Class of 2025

Thank You

DEDICATIONS

First and foremost, I would like to praise God and be grateful for the health and strength granted throughout the research project, which allowed me to finish the research project successfully without any trouble.

I want to extend my deepest gratitude towards my loving parents, Chitra Iyyadurai, who always gave me words of encouragement and utmost support throughout this process. My siblings have always been with me and provided any means necessary to help, leading me to where I am today.

Next, I would also like to dedicate this dissertation to my supervisor, Assoc. Prof Dr. Rumaizi Bin Shaari and my co-supervisor, Ts. Dr. Muhammad Luqman Bin Nordin, for their unconditional support and guidance. Special thanks also to Ts. Dr. Muhammad Luqman Bin Nordin for allowing me to use his materials required for the research project and guiding me patiently during the process, which led to the completion of this thesis. Special thanks to Dr Roslina binti Rashid for her unwavering support throughout the duration of the experiment. I would also like to dedicate this work to Dr. Fathin Faahimaah binti Abdul Hamid, for her great help and support in pathology.

Finally, I would also like to dedicate this work and give special thanks to my DVM 3 junior Eii Tze Min and DVM 5 rotation group mates, Izzah Binti Yusof, Nur Hanani Binti Mohamed Haris, Nurul Najiha Binti Ahmad Jame, Nik Ameera Syadiyah Binti Azhar, and Leong Zhi Zong for a being there to help me at a moments notice and supporting me endlessly throughout the research project.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

GP - *Gynura procumbens*

ADT - Acute Dermal Toxicity

C - Chitosan

PVA - Polyvinyl Alcohol

SA - Sodium Alginate

ALT - Alanine aminotransferase

AST - Aspartate aminotransferase

CREA - Creatinine

SDR - Sprague Dawley Rats

RBC - Red Blood Cell

H&E - Hematoxylin and Eosin Stain

CHAPTER 1

1.0 Introduction

Skin serves many purposes. It serves as a barrier against pathogens and water loss and offers resistance against a variety types of trauma, such as thermal, chemical, and UV radiation. (Abdo *et al*, 2020) Through a diverse range of nerve endings, skin keeps us aware to our surroundings, controls body temperature, improves metabolic processes, and integrates vitamin D. (Chuong *et al.*, 2002) Skin needs three associated structural properties to achieve this goal – Layered interface, epidermal appendage, and mechanical solidity (Abdo *et al*., 2020). Ergo, detrimental skin injury may endanger life. Skin wound recovery demonstrates an exceptional, unique cellular function mechanism. Cells, growth factors, and cytokines work together during the repair process to heal the lesion (Tottoli *et al.,* 2020). The generation of a viable blood vessel network by angiogenesis is a vital step in the wound recovery process. Angiogenesis enables the restoration of regular blood flow, adequate oxygen and nutrient transport, and the elimination of metabolic waste, all of which are essential for cell growth and maintenance (Guerra *et al*., 2018).

Wound dressings are valuable tools in wound healing management. Different dressings can be worn to the wounded areas to encourage healing depending on the types and degrees of the wound's severity. (Shi *et al*., 2020) Given that modern dressings have qualities that provide a moist environment for wound healing, they may be more worthy candidates. (Gupta *et al*., 2018) Modern dressings have superior biocompatibility, degradability, and moisture preservation in comparison to previous dressings which are mainly inert. The benefits of modern dressings include pain relief and an enhancement in the anaerobic or hypoxic environment. (Han *et al*., 2023) In clinical settings, hydrogels, hydrocolloid, alginates, foams, and films are the most often utilised current dressings (Shi *et al*., 2020).

Gynura procumbens (GP) also known as Longevity spinach and Akar Sebiak locally in Malaysia is a herbal flowering plant that grows up to 1-3 meters in height and is found in multiple countries across South East Asia. Traditionally, it has long been utilized in folk medicine to cure a variety of conditions, such as diabetes, kidney stones, hypertension, and inflammation in humans. (Sutthammikorn *et al.*, 2021) The *GP* leaves yields diversified phytoconstituents including different macroelements and microelements, alkaloids, saponins, chlorophyll, carotenoids, essential oils, etc (Mou *et al*., 2016) With more modern research advancing into health benefits of traditional treatment, the potential health benefits that can emerge from the plant is exponential.

1.1 PROBLEM STATEMENT

The treatment and mitigation of diseases in both humans and animals are largely facilitated by medicines. The potential environmental implications of the production and use of pharmaceutical products, however, are not fully understood, despite the fact that adverse reactions on human and animal health are typically comprehensively evaluated in safety and toxicity assessments. Unexpected environmental consequences could result from disintegrated disposed drugs and the mixing of several biologically active substances.

The term "medical waste" is used to represent any solid or liquid waste that is produced during the diagnosis, management, or immunization of humans or animals, as well as during related research, the creation or analysis of biopharmaceuticals, or any of these activities. (The 1988 Medical Waste Tracking Act) Due to the presence of highly hazardous compounds and dangerous sharp structures, it has been demonstrated in several studies that medical waste can serve as a possible source of poisoning and act as a pathway for the spread of serious health issues. (Bolan *et al*., 2023)

Besides bypassing metabolic functions and ameliorating patient's compliance, topical dosage form diminishes the necessity for invasive, unpleasant needles that emit medical waste, the raise of infection likelihood, and the requirement for a treatment to be performed by qualified medical personnel. Therefore, the modelling of polymer-based film immersed with natural sources is brought into focus to minimize environmental consequences.

Medicinal herbs have proven to help in various fields of medicine as alternate medicine to combat pathogens and to be used as anti-inflammatory. (Alanazi *et al*., 2023) *GP* is a plant that is boasted to have medicinal properties that assist in wound healing. However there is no research done on proving the properties of the plants will be useful in wound healing. The study aims to use *GP* extract to determine any Acute Dermal Toxicity (ADT) based on current polymer films that are available in the market to help patients in the future.

1.2 RESEARCH QUESTION

1. Does *Gynura procumbens* cause Acute Dermal Toxicity?

2. Does *Gynura procumbens* have negative effects towards liver and kidney?

1.3 RESEARCH HYPOTHESIS

- *Gynura procumbens* extract will not cause any ADT.

- There will be no deleterious effects of using *Gynura procumbens* as an alternate medication on liver and kidney blood parameters.

1.4 RESEARCH OBJECTIVES

1.4.1 General Objective

To formulate and construct various biopolymer films encompassing chitosan, polyvinyl alcohol, and sodium alginate infused with *Gynura procumbens* extracts as a development of antibacterial wound dressing.

1.4.2 Specific Objective

1. To assess the ADT effect of biopolymer films loaded with *Gynura procumbens* extracts in Sprague Dawley Rats (SDR).
2. To assess the behaviour of SDRs before and after induction with biofilms.
3. To assess the histopathology and blood parameters of kidney and liver in SDR treated with *GP* biofilm.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 WOUNDS

A wound is an obstruction in the structure and function of fundamental skin tissue within the skin's epithelial structure. There are multiple roots of wounds. Cutting, abrasion, surgery, injuries, and burns are the primary causes of cuts and bites (Sharma *et al*., 2020). Depending on the underlying etiologies and effects of these forms of damage, they are characterised either as acute wounds or chronic wounds. (Bowler *et al*., 2001) Acute wounds often perform a coordinated and efficient recovery process, leading to the long-term restoration of anatomical and functional viability. On the other hand, chronic wounds are remarkable for preserving the best possible anatomical and functional integrity (Tottoli *et al.*, 2020).

Inadequate wound repairs can lead to major harm, including skin loss and the propagation of an infection, which can injure nearby tissues as well as systemic ones. (Wernick B *et al*., 2024) The initiation of an infection, most notably in the event of chronic wounds, is the most prevalent and unavoidable barrier to wound healing. (Bowler *et al*., 2001) Although bacteria are a normal component of wounds and the intact skin microbiota, a serious level of bacteria present and the generation of a biofilm may hinder wound healing. (Percival *et al*., 2011) For these circumstances, bacterial and fungal infections are still regarded as among the most widespread and painful conditions that cause significant mortality and morbidity, despite recent advancements in the management of wounds (Negut *et al*., 2018).

2.2 *Gynura procumbens*

In the countries of tropical Asia, Gynura procumbens is a widely known plant utilized in traditional medicine. (Tan *et al.*, 2016) The plant belongs to Asteraceae-family and locals from nations such as China, Vietnam, Malaysia, Indonesia, and Thailand frequently employ the benefits of this plant to treat diabetes, cancer, hypertension, inflammation, fever, and skin diseases (Amin *et al.*, 2021). Gynura procumbens was formerly known as "Sambungnyawa," a plant whose name also implies everlasting life. It is sometimes referred to as "diabetes herb", "Sambungcho", (Kim *et al*., 2021) and “Bai Bing Cao” as well. This is due to the fact that it has been used in traditional medicine to treat a variety of illnesses both systemically and topically (Tan *et al.*, 2016). Both raw and cooked Gynura procumbens are safe to eat on a regular basis. They taste mildly raw, have little flavour, and faintly pungent fragrance (Adeib Idris *et al.*, 2019).

2.3 MEDICINAL ACTIVITIES OF *Gynura procumbens*

2.3.1 COX-2 Ligand

The pathway known as COX-2 is expressed in response to growth factors, inflammatory stimuli, and other physiological stimuli. (Simon, 1999) It plays a role in the synthesis of prostaglandins, which are implicated in pain mediation and the promotion of inflammation. (Ricciotti & FitzGerald, 2011) Compounds contained in the plant extract mainly caffeic acid, kynurenic acid and chlorogenic acid functioned as COX-2 ligands successfully by binding specifically with COX-2, reducing the conversion of arachidonic acid, inhibiting inflammatory mediators such as prostaglandins, leukotrienes, and cytokines. (Qureshi O *et al.,* 2024)

2.3.2 Reduction of Anti oxidative stress

Oxidative stress occurs when there is an imbalance between free radicals and antioxidants in the body. (Pizzino *et al*., 2017) Free radicals are produced by the cells as part of regular metabolic processes. (Pizzino *et al*., 2017) Antioxidants are also created by the body or consumed from external sources that neutralise these free radicals. The body will always attempt to create homeostasis by maintaining a balance of antioxidants and free radicals. (Phaniendra *et al*., 2014) *Gynura procumbens* extract avoided damage to membrane cells by altering the lipid profile. These findings proposed that the major phenolic contents, chlorogenic acid, gallic acid, kaempferol, quercetin, and rutin, identified in *Gynura procumbens* extract were potent antioxidants and scavengers of ROS (Nazri *et al*, 2021)

2.3.3 Angiogenesis

Wound healing process consists of 4 phases that occur in a timely manner and correct sequence to return tissue normal function. (Wallace HA *et al.,* 2023*)* Several of the various phases may be influenced by a variety of conditions, which will ultimately affect how the healing process completes.(Schultz GS *et al*., 2021) It is believed that Gynura procumbens initiates angiogenesis on the wound, drastically reducing the healing time needed. Overall, *G. procumbens* shortened the wound healing time in healthy and diabetic mice by 30% and 40%, respectively. (Sutthammikorn *et al.,* 2021)

2.4 CHITOSAN

The natural polymer chitosan is made from chitin, which is extracted from the shells of crustaceans including lobster, crab, and prawns. (Sharkawy *et al.*, 2020) It is a material that is both biocompatible and biodegradable, and has a broad variety of uses, especially in the food, pharmaceutical, cosmetic, and agricultural industries. (Morin-Crini *et al.*, 2019) Chitosan is highly biocompatible; it does not lead to allergic reactions or rejection and is degraded to nontoxic amino sugars in tissues. (Herdiana *et al.*, 2021) Chitosan nanoparticle is a drug carrier with the some advantage of slow and controlled drug release, which improves drug solubility and stability, efficacy and reduces toxicity (Herdiana *et al.*, 2021). Due to its specific and distinctive properties, including its low toxicity, biodegradability, biocompatibility, and non-immunogenicity, chitosan is an excellent drug delivery system.

2.5 SYNTHETIC AND NATURAL POLYMERS

The development of modern medicine has been heavily influenced by the incorporation of polymers as biomaterials. (Ulery *et al.*, 2011) Particularly, biodegradable polymeric biomaterials have considerable benefits of being able to be decomposed and eliminated after they have completed their intended purpose (Ulery *et al.*, 2011). Green chemistry and eco-friendly engineering are being recognized as a key option for the improvement of the upcoming new line of products, materials, and processes as a rebuttal to the emergence of comprehensive environmental issues, philosophies of sustainability, production ideologies, and ecosystem efficacy (George *et al.*, 2020). Biopolymers offer non-toxicity, non-antigenicity, inertness, bio adhesiveness, biocompatibility, biodegradable, efficacious hemostatic impacts, and antibacterial attributes as advantages. (Alven & Aderibigbe, 2021) However, biopolymers exhibit inadequate mechanical characteristics while synthetic polymers lack biocompatibility. Therefore, hybrid polymers with desirable physicochemical properties can be constructed by combining both synthetic polymers and biopolymers (Alven *et al*., 2020).

The terminology "biopolymers" denotes materials that are essentially made from living things, as indicated by the prefix "bio". (Liu *et al*., 2023) Biopolymers have found their way into the world of materials and are composed of chain-like organised molecules that are either linearly arranged or branched/cross-linked. (Ottenbrite & Javan, 2005) Most biopolymers have repeating monomer units composed of either nucleic acid of nucleotides, amino acid proteins, or saccharides generated from sugars (George *et al*., 2020) which in turn, becomes the basis of how natural polymers are classified (Reddy *et al*., 2021)

Table 1: Examples of natural polymers (Yannas *et al*., 2004)

Class	Example
Polynucleotide-based	Linear plasmid DNA, DNA, RNA
Polypeptide-based	Collagen, fibrin, fibrinogen, gelatin, silk, elastin, myosin, keratin, actin
Polysaccharide-based	Chitin, chitosan, alginate, hyaluronic acid, cellulose, agarose, dextran, glycosaminoglycans

Moreover, polyethylene glycol (PEG) or polyethylene oxide (PEO), polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), polyhydroxy ethyl methacrylate (PHEMA), polyurethanes (PUs), and poly (α-esters) such as polylactic-co-glycolic acid (PLGA), polyglycolic acid (PGA), polylactide (PLA), and poly(ε-caprolactone) (PCL) are among the synthetic polymers utilized in wound dressing formulations (Alven *et al*., 2020).

Despite the fact that natural polymers like collagen have been exploited in medicine for thousands of years, the purposes of artificial degradable polymers in the biomedical field have just recently been studied, with investigations beginning in the 1960s. (Nair & Laurencin, 2007) There have been many breakthroughs over the past 50 years, yet there are still significant obstacles to overcome in the basic and translational aspects of biomaterial innovation. From a basic scientific standpoint, the ability to manipulate biomaterial chemistry to transmit specific material features is limitless but doing so takes a large investment in time and money. (Ulery *et al.*, 2011).

2.7 FILM FORMULATION

The creation of polymeric materials for diverse environmental functions has advanced significantly. (Arif *et al*., 2022) Over the past few decades, these developments in the material sciences have led to astounding discoveries in route-specific drug carrier systems particularly in transdermal drug delivery systems (Sivasankarapillai *et al*., 2021). The strengths of films and hydrogels can be integrated to manufacture practical and useful film-forming devices to control medication administration through the skin. (Thang *et al* ., 2023) Considerable approaches have been established to modulate drug delivery through the skin by incorporating alterations to plasticizers, additives, film-forming polymers, and even model drugs in formulations (Tran & Tran, 2019).

Local and systemic therapeutic effects can be attained using transdermal administration of medication. (Badilli *et al*., 2018) In contrast to oral dosage form, transdermal drug delivery avoids a multitude of challenges, including first-pass biotransformation, enzymatic metabolism, drug hydrolysis in acidic conditions, gastrointestinal irritation, drug perturbations, adverse effects and therapeutic failure, and the risk of disease transmission. (Homayun *et al.*, 2019) Additional benefits include controllable drug release, cheap cost, and better patient acceptance. (Adepu & Ramakrishna, 2021) Some drawbacks of transdermal drug delivery system include the potential for skin irritation, the inability to distribute ionic agents, and the inadequacy of the method for patients who are in shock or have poor peripheral blood flow (Al Hanbali *et al*., 2019).

After topical administration, films provide the capacity to sustain prolonged steady-state blood levels. (Adepu & Ramakrishna, 2021) The levels can be adjusted by varying the drug concentration, the vehicle components employed in the patch, and/or the surface area of the skin in contact with the patch. (Adepu & Ramakrishna, 2021) Given that therapeutic blood levels would need to be reached, the drug's potency was emphasized as a crucial therapeutic factor (Pastore *et*

al., 2015). Drugs with poor partition coefficients will have their dermal and transdermal distribution boosted by the use of prodrugs. The prodrug design incorporates the inclusion of promoiety to promote the parent drug's stratum conium transport, solubility, and partition coefficient (Bathe & Kapoor, 2015).

According to their composition, transdermal film formulations can be divided into two types (Sivasankarapillai *et al*., 2021) :

Table 2: Classification of transdermal films (Sivasankarapillai *et al*., 2021)

Types of Transdermal Film	
Matrix	**Reservoir**
- Drug, adhesive, and a mechanical support structure compose its simple design. - The drug is simpler to assemble since it is encapsulated in a polymer network matrix. - Rate controlling membrane is absent and its flexibility is inadequate. - Skin permeability indicates the rate of drug release.	- Complicated design. - Active ingredients are retained in a solution or gel state and the release of them is permitted by a rate-controlling device positioned between the reservoir and the tissue. - There are noticeable initial burst rates. - Provides greater control over administration rates and more formulation versatility.

Membrane matrix type hybrid patch is a modified type of reservoir transdermal patch. The distinctive contrast is the placing of a solid polymer between the backing laminate and rate-controlling membrane instead of the drug reservoir's liquid formulation.

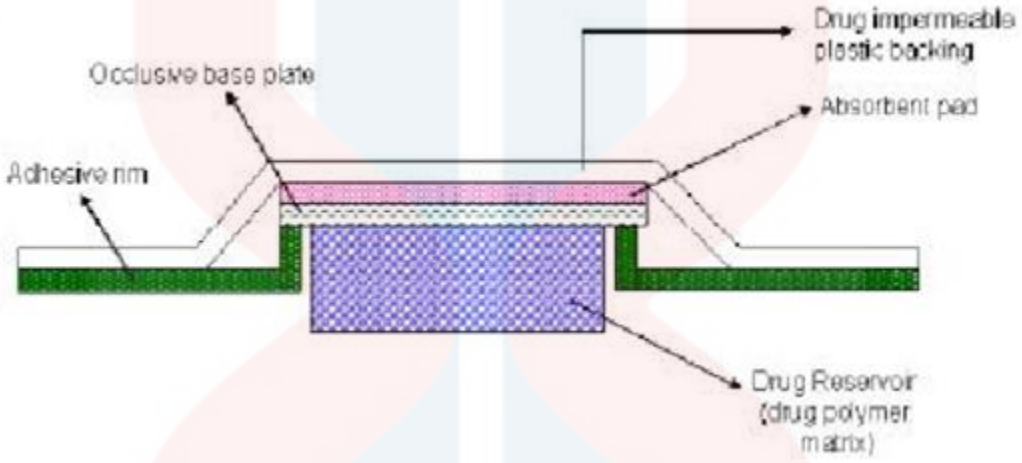


Figure 1: Show the composition of membrane matrix type hybrid patch. (Wahghulde *et al.*, 2013*)*

CHAPTER 3

3.0 RESEARCH METHODOLOGY

3.1 MATERIALS AND METHODS

The need for accessing the properties of GP on live animal models requires a variety of planning on the needed materials and methods to ensure accurate and ethical testing. GP leaves were sourced locally, dried and ground before being used for the extraction process. Extraction of compounds from the plant requires specialised equipment to synthesize an extraction that is suitable to be used in the investigation that is discussed further in the paper. The biofilms that were used as vehicles in the study were engineered to be non toxic and stable as a drug delivery system. The enhancement of the biofilm mechanical strength through mixing of compounds fulfills the requirements to be used in biological systems. (Enoch *et al*., 2023) The study was conducted following the OECD 402 Guidelines in Animal Research Lab, FPV UMK. The primary target of the investigation is to assess the skin, kidney and liver in terms of toxicity effects that can be caused by the biofilms infused with GP. The use of healthy Sprague Dawley Rats is necessary to eliminate disease factors that could contribute to changes in the results. (Laaldin *et al*., 2019)

3.1.1 PREPARATION OF LEAF EXTRACT

The extraction of *Gynura procumbens* leaves was carried out by employing the Soxhlet extraction method. An altered figure based on Shamsuddin, 2015 has been made to demonstrate the Soxhlet extraction method (Figure 2). At a ratio of 1:20 (w/v), 1000 mL of 75% ethanol extracted 50 g of dried powdered leaves (Nazri *et al.*, 2021). During this procedure, the solvent was vaporized and condensed on the powdered sample placed in the thimble so that the compounds soluble in ethanol were separated. The solvent containing extracted compound was then siphoned back into the distillation flask once it reached the top of the extractor. The acquired crude extract was further concentrated using a vacuum rotary evaporator to eliminate the remaining solvent after 6 hours of Soxhlet extraction. Extracts were allowed to dry in the oven for a few days before being kept in an airtight bottle at -4 °C (Katare *et al.*, 2022).

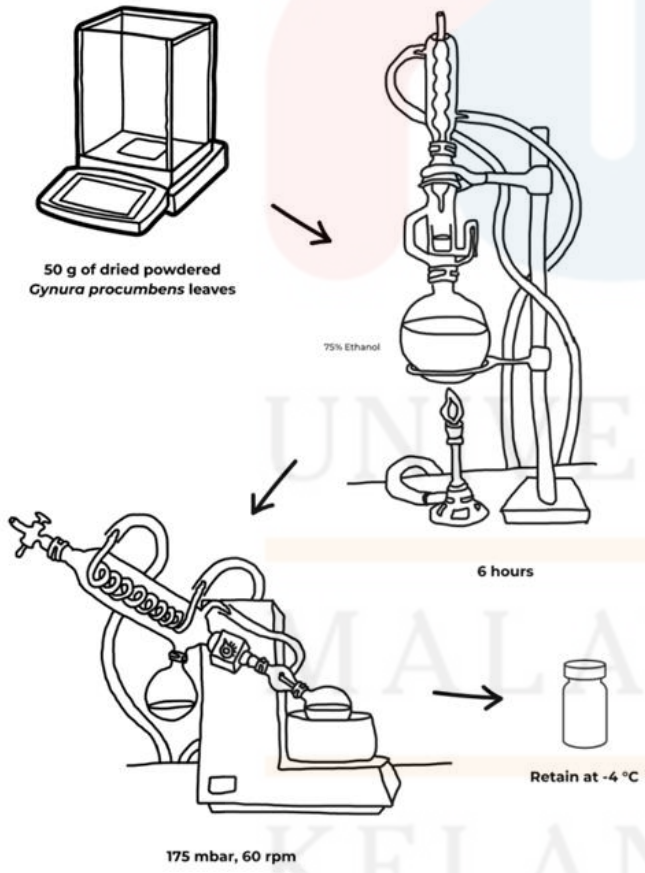


Figure 2: Soxhlet extraction method (Shamsuddin *et al.*, 2015)

3.1.2 PREPARATION OF FILM FORMULATION

CH/PVA-based Film Infused with *Gynura procumbens* Extract

A solvent-casting technique was adopted to develop a hydrogel film consisting of chitosan and PVA (F1). Chitosan was dissolved in a 2% acetic acid solution while being continuously stirred at 60 °C until the solid matter is completely dissolved to produce chitosan solution (3% w/v). PVA was dissolved in distilled water with consistent stirring at 85 °C until a clear solution was acquired to create PVA solution (5% w/v). The chitosan and PVA solutions were then merged mechanically with *Gynura procumbens* extract (2% w/v) for 10 minutes at 1000 rpm. The resulting mixture was poured into petri dishes and kept two days in the oven at 40 °C to dry. The dried films were then removed from the petri dishes and preserved for further use in a desiccator (Chopra *et al.*, 2022). Figure 3 shows an altered graphic representation based on Qureshi *et al.*, 2021 for the assembly of CH/PVA films.

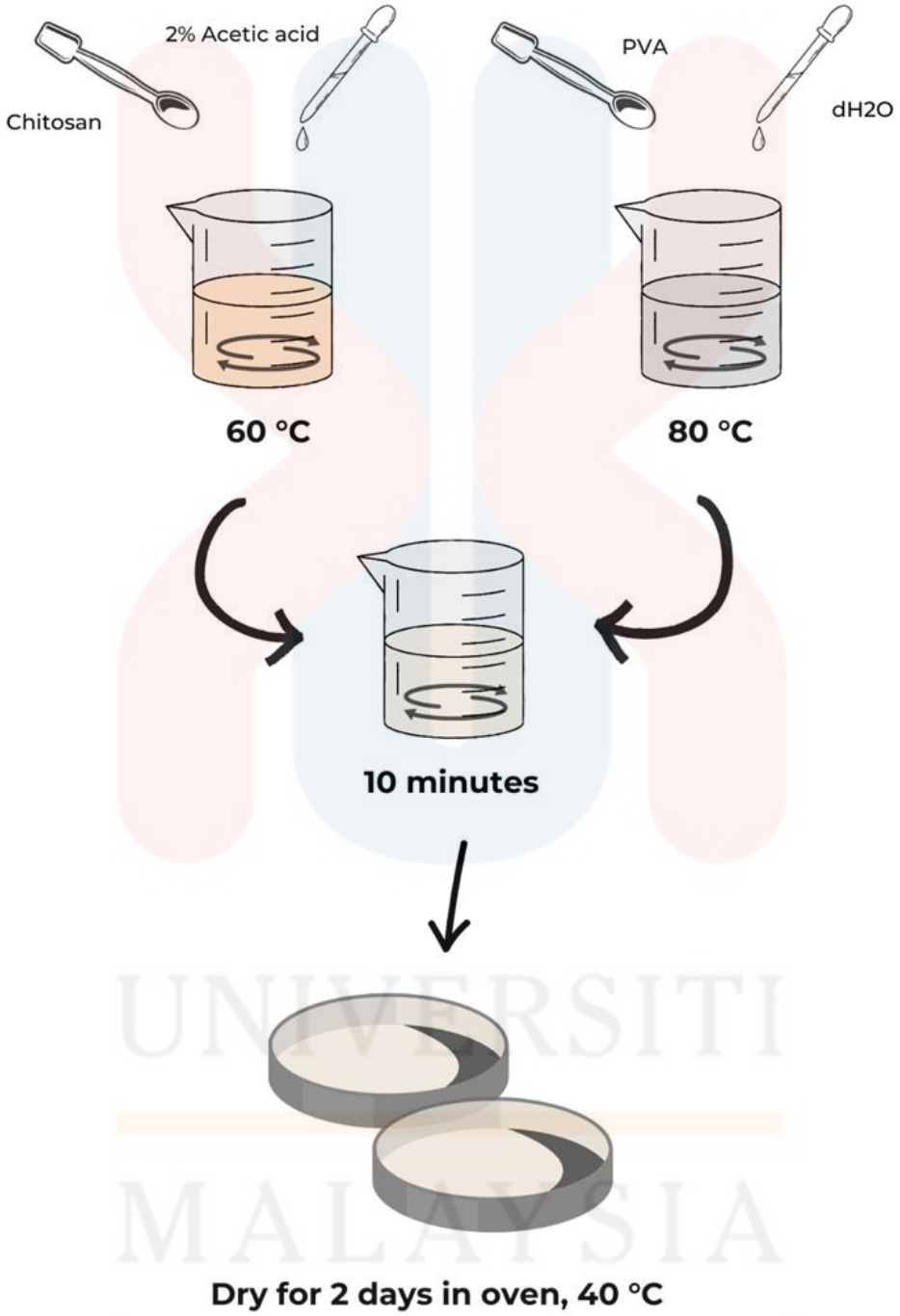


Figure 3: Preparation of Chitosan/PVA film (Qureshi *et al.*, 2021)

CH/PVA/SA-based Film Infused with *Gynura procumbens* Extract

Chitosan, PVA, and sodium alginate were combined to form a hydrogel film (F2) by implementing solvent-casting technique as well. Sodium alginate solution (3% w/v) were firstly obtained by mixing distilled water with alginate powder and stirring at 70 °C until a homogenous gel was produced. With constant stirring and temperature, 3 g of PVA were introduced to the gel. Chitosan powder was combined with 1% acetic acid in another beaker to form chitosan solution (1.5% w/v). The resulting mixture was stirred at 60 °C until all the suspended chitosan disappeared. As a plasticizer, another 3 g of PVA was added to this solution and thoroughly stirred until a uniform dispersion is developed.

Next, the CH/PVA solution was transferred into the SA/PVA solution drop by drop. After both solutions have blended, 2 g of *Gynura procumbens* extract was then introduced. This step was performed using a probe sonicator for 30 minutes at a 30% amplitude in an ice bath (Janardhanam *et al*., 2020). For another 30 minutes, stirring was then carried on with a stirrer. The gel was laid out in a petri dish, and the films were dried in the oven at a temperature of 40 °C for 2 days. The solidified films were then subsequently peeled from the petri dish and kept in the desiccator. A biofilm (F3) was also created by increasing the mixed compounds to make a mixture of 3% Chitosan-PVA-SA. An altered visual illustration of the construction of hydrogels is shown in Figure 4 based on Farazin *et al.*, 2021.

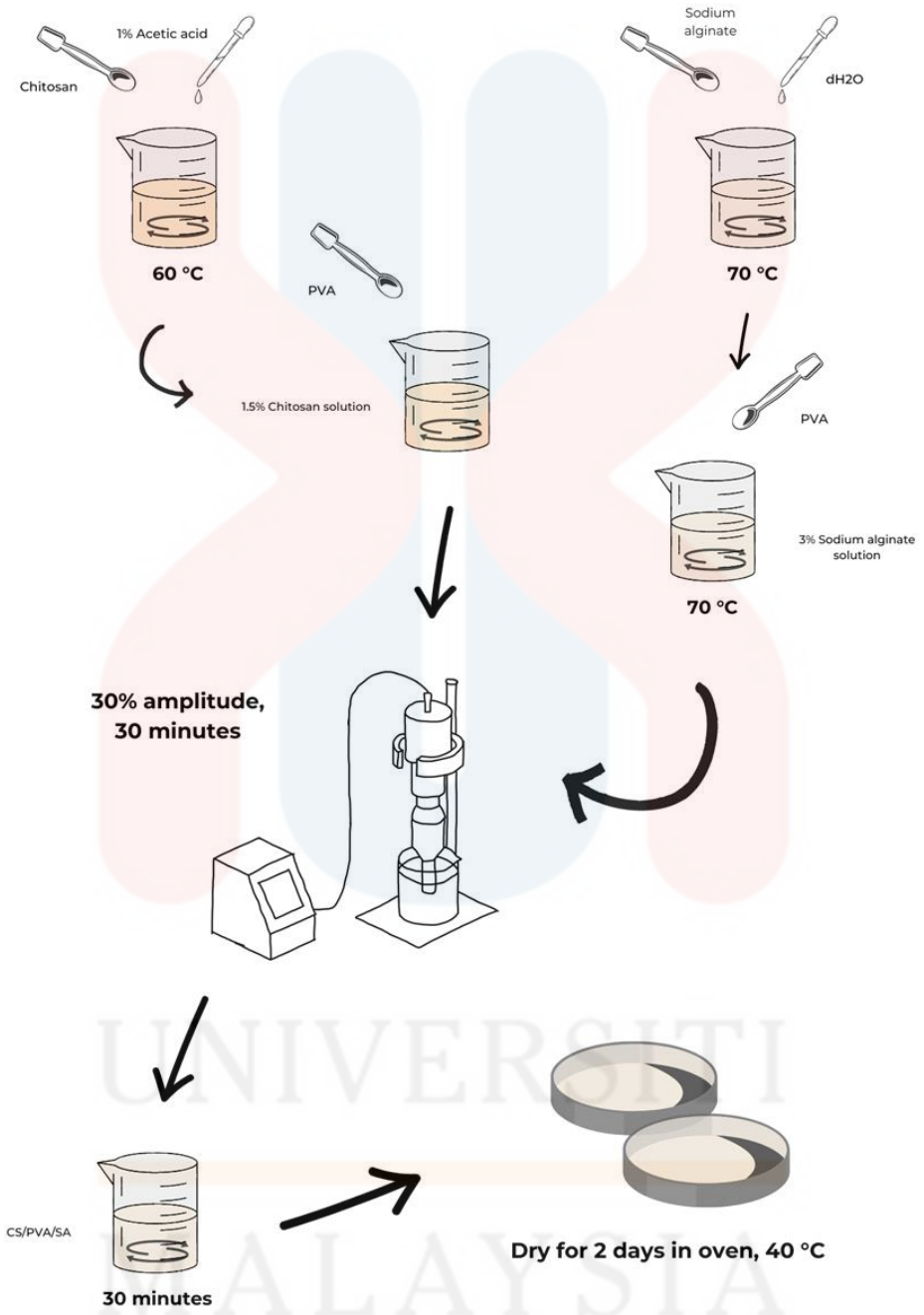


Figure 4: Preparation Chitosan/PVA/Sodium alginate film (Farazin *et al*., 2021)

3.2 ETHICAL CONSIDERATIONS

Animal ethics have been applied and approved by the Animal Ethics Committee prior to the start of the experiment. Animal Ethics Approval Code: UMK/FPV/ACUE/FYP/001/2024

3.3 EXPERIMENTAL ANIMALS

Inclusion criteria of experiment for reliable, reproducible and ethical results based on ARRIVE 2.0 guidelines. (Du Sert *et al.*, 2020) Sprague Dawley rats weighing 250–300g were used for the study at age 7-8 weeks old. The healthy rats were obtained from Animal Research and Service Centre (ARASC), Universiti Sains Malaysia in Kubang Kerian, Kota Bharu, Kelantan. The rats were male sexed to avoid interruption to the study due to hormonal changes that occur in the female sex. (Hinchcliffe *et al.,* 2020) The rats were marked on the tail using animal crayon for identification purposes. (Mazlan *et al.*, 2014) Animals are healthy with no physical signs of disease or trauma present. Animals will be excluded if any abnormality is presented before the initialisation of the experiment. Abnormality that is included but not limited to is behavior, prior treatment, congenital issue and visible sign of disease. (Du Sert *et al.*, 2020) The rats were kept in the Research Animal Laboratory room of Faculty of Veterinary Medicine, Universiti Malaysia Kelantan under controlled environment for a period of 5 weeks.

3.4 HOUSING AND FEEDING CONDITION OF EXPERIMENTAL ANIMALS

The rats were housed at the Animal Research Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan (UMK). All the rats were housed in 3 cages sized 545*395*200 mm (Length*Width*Height) with 5 rats per cage and kept under a controlled temperature (27 °C ± 2 °C), 12 h light/12 h dark conditions. Their diet was Rat Block 702P Gold Coin with unlimited supply of Coway filtered clean water via bottle drinker. The animals were subject to care based on regulated animal welfare laws.

3.5 EXPERIMENTAL DESIGN

Sample size is calculated using the "resource equation" method (Charan & Kantharia, 2013). According to this method a value "E" is measured, which is the degree of freedom of analysis of variance (ANOVA). The value of E should lie between 10 and 20; Any sample size, which keeps E between 10 and 20 should be considered as adequate.

$$E = \text{Total number of animals} - \text{Total number of groups}$$

Suppose we want to make 3 groups consisting of 5 rats each. In this case E will be:

$$E = (3 \times 5) - 5$$

$E = 15 - 5 = 10$, which is in the range of 10 to 20, hence sample size in this experiment can be considered as adequate sample size.

3.6 ACUTE DERMAL TOXICITY TEST

The *in vivo* acute dermal toxicity tests were conducted according to the Organization for Economic Cooperation and Development (OECD 402) guideline *in vivo* acute dermal toxicity.

Animals were first acclimated for a period of 14 days in the Animal Research Laboratory. Animal were anesthetised with a cocktail of Ketamine + Xylazine based on Anesthesia guideline of University of Iowa, reviewed by the IACUC. Animals were then shaved dorsal of the abdomen extending to the right hind limb and perianal region. The skin was shaved using a razor blade to help the biofilm adhere better to the skin.(Figure 5) The procedure is done in accordance to the USDA Policy #11. The testing procedure is categorised under USDA Category C where there is slight and momentary distress due to handling which is relieved by anesthesia. Biofilm for each group is stated Table 3. The biofilm was placed on the shaved skin followed by Oxymecx Non-adhesive absorbent pad and finally with Fixomull Adhesion tape. To provide extra security, the Fixomull tape was covered with coban and adhesive tape. (Figure 6 Appendix) Animals were observed immediately after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 2 to 6 hours after the beginning of the exposure period, and daily thereafter, for a total of 14 days. (OECD 2017) The biofilm and dressing was removed after 24 hours to observe for adverse dermal reactions using the Draize Dermal Irritation Scoring System. (Hemmati *et al.*, 2016)

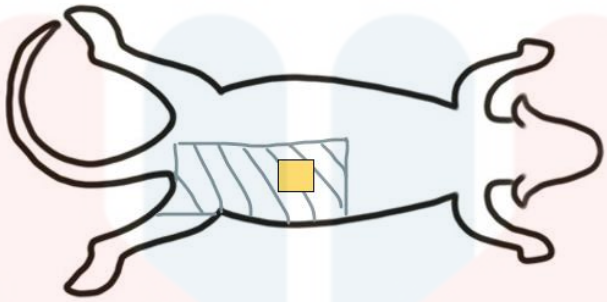


Figure 5: Showcase of trimmed hair and position of biofilm

Group	A	B	C
Biofilm	3% Chitosan-PVA + *Gynura procumbens* extract	1.5% Chitosan-PVA-SA + *Gynura procumbens* extract	3% Chitosan-PVA-SA + *Gynura procumbens* extract

Table 3: Grouping of biofilm

Erythema and Eschar Formation	Value	Edema Formation	Value
No erythema	0	No edema	0
Very slight erythema (barely perceptible)	1	Very slight erythema (barely perceptible)	1
Well-defined erythema	2	Slight edema (edges of area well defined by definite raising)	2
Moderate to severe erythema	3	Moderate edema (raised approximately 1 mm)	3

Table 4: Draize Dermal Irritation Scoring (Hemmati *et al.*, 2016)

3.7 SERUM BIOCHEMISTRY TEST

At the end of the experimental period, the animals were euthanized with carbon dioxide gas and blood samples of 2 ml were taken via cardiac puncture phlebotomy and stored in the whole blood tube. Serum was used in biochemical investigations. (Delwatta, 2018). All parameters are cross referenced to existing established parameters available for *Rattus norvegicus.*

Serum was assessed by the University Veterinary Diagnostic Center, FPV UMK. The serum was isolated and free from platelets by centrifugation at 2000 rpm for 10-15 min before used for biochemical assessment. The parameters that were targeted for this project include Alanine transferase (IU/L), Aspartate aminotransferase (IU/L), Urea (mmol/L), and Creatinine (μmol/L).

3.8 HISTOPATHOLOGY

The diagnosis and research of disorders affecting the tissues require microscopic examination of tissue and/or cell structure and any distinctive alterations the tissue and/or cell may have undergone. The following procedures were taken in order to prepare tissue samples: fixation, processing, embedding, and sectioning. (Figure 12 Appendix) Segments of tissue from skin, kidney and liver were fixed in 10% formalin to be further processed according to the standard procedures of the histopathology lab. (Figure 11 Appendix) Samples were stained with hematoxylin and eosin stains and changes were observed under microscope with photographic facility (3DHISTECH) and their pictomicrograph were taken.

Pictomicrographs were scored for presence of inflammatory cells using Inflammatory Cell Scoring system adapted by Gibson-Corley *et al*., 2013. 4 views were observed for each slide and the total number of inflammatory cells were added to determine the score for each sample.

Number of Inflammatory Cells (x40 magnification)	Score	Description
0-5	1	Absent
6-20	2	Scarce
21-50	3	Moderate
51-150	4	Abundant
>150	5	Severe

Table 5: Histopathological IC scoring. (Gibson-Corley *et al*., 2013)

3.9 STATISTICAL ANALYSIS

Creatinine, Urea, Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) were statistically analyzed using GraphPad Prism 10.0. Normality of distribution was confirmed with the Shapiro Wilk test. Statistical analysis done using One Way Anova and Kruskal-Wallis with 95% confidence interval, where $p<0.05$ was considered as significant. Comparisons between the 3 treatment groups were done with Tukey's and Dunn's multiple comparisons test. All data were expressed as mean±standard error of the mean.

Histopathological findings were statistically analyzed using GraphPad Prism 10.0. Normality of distribution was confirmed with the Shapiro Wilk test. Statistical analysis done using Kruskal-Wallis with 95% confidence interval, where $p<0.05$ was considered as significant. Comparisons between the 3 treatment groups were done with Dunn's comparisons test.

CHAPTER 4

4.0 RESULTS

One of the most popular inbred strains of laboratory rats, especially for toxicological studies, are Sprague-Dawley (SD) rats. Their widespread use is explained by a number of factors, such as their well-established behaviour, physiological traits, and genetic background, which make them appropriate for a range of experimental investigations. (Foster & Frost, 2017) Sprague-Dawley rats were selected because of their extensive use in medical stability testing, which should make it easy to compare the data from this study with those from numerous other databases. (Son *et al.*, 2014)

4.1 ACUTE DERMAL TOXICITY

During the initial 24 hour period of induction, all rats showed no significant changes or impairment in their behavior. The removal of the bandages after 24 hours showed an increased non- intake maintenance of self grooming among all 3 groups of rats for the first 12 hours. On Day 5 Post induction, 1 rat from Group C was found dead in the morning with no gross abnormalities. No other abnormalities in behavior were noted for the following period of 13 days. Behaviour observation was scored based on ethogram by Abou-Ismail *et al.*, (2007).

The ADT was also scored using the Draize Dermal Irritation scoring system. (Hemmati *et al*., 2016)

Group A: 2 rats developed erythemia (¼) that resolved in 2 days, 1 rat developed erythemia (2/4) that resolved in 6 days.

Group B: 1 rat developed erythemia (¼) that resolved in 3 days, 1 rat developed erythemia (2/4) that resolved in 5 days.

Group C: 1 rat developed erythemia (¼) that resolved in 2 days, 1 rat developed erythemia (2/4) that resolved in 5 days.

4.2 SERUM BIOCHEMISTRY ANALYSIS

Panel	Reference Range	A	B	C
UREA	9-21 mg/dl	18.9±1.355	18.88±1.052	18.87±0.376
CREA	0.05-0.65 mg/dl	0.3780±0.041	0.4520±0.044	0.4000±0.040
ALT	20-61 u/l	61.42±1.301	60.64±0.702	59.77±0.404
AST	39-111 u/l	79.58±1.975	78.80±1.693	80.67±0.577

Table 6: Mean results of serum biochemistry. The value is expressed as mean ± Standard Deviation.

The statistics analysis used is Parametric One Way ANOVA and Tukey's multiple comparison tests with significance level ($p<0.05$) for the sample were normally distributed. Non Parametric, Kruskal Wallis and Dunnett's multiple comparison tests with significance level ($p<0.05$) were conducted for samples that are not normally distributed. Table 6 manifests the mean values of each of the biofilm groups after ADT study. The mean biochemistry panels are still within the reference range for all the groups.

The mean of Urea, Creatinine, ALT and AST after toxicological study using Chitosan/PVA/SA + *Gynura procumbens* in 3 different biofilms was analyzed and tabulated in a bar chart.

The bar chart shows the mean values between the 3 groups. Based on the figure the Minimum Urea value is 16.80mg/dl and maximum value 20.10mg/dl, with a median of 19.60mg/dl. One way ANOVA analysis suggests that urea value does not differ significantly ($p > 0.05$).

The Creatinine minimum value is 0.32mg/dl and maximum value 0.42mg/dl, with a median of 0.40mg/dl. One way ANOVA analysis suggests that creatinine value does differ significantly across the three biofilms ($P<0.05$). Tukey's multiple comparison analysis suggests that the mean of Creatinine between Group A and B differ significantly.

The ALT minimum value is 60.00µ/l and maximum value 63.00µg/l, with a median of 61.20µg/l. Kruskal Wallis analysis suggests that ALT value does differ significantly across the three biofilms ($P < 0.05$)

AST values between the 3 groups. Based on the figure the Minimum value is 76.30µ/l and maximum value 81.00µg/l, with a median of 80.40µg/l. Kruskal Wallis analysis suggests that AST value does not differ significantly across the three biofilms ($P > 0.05$)

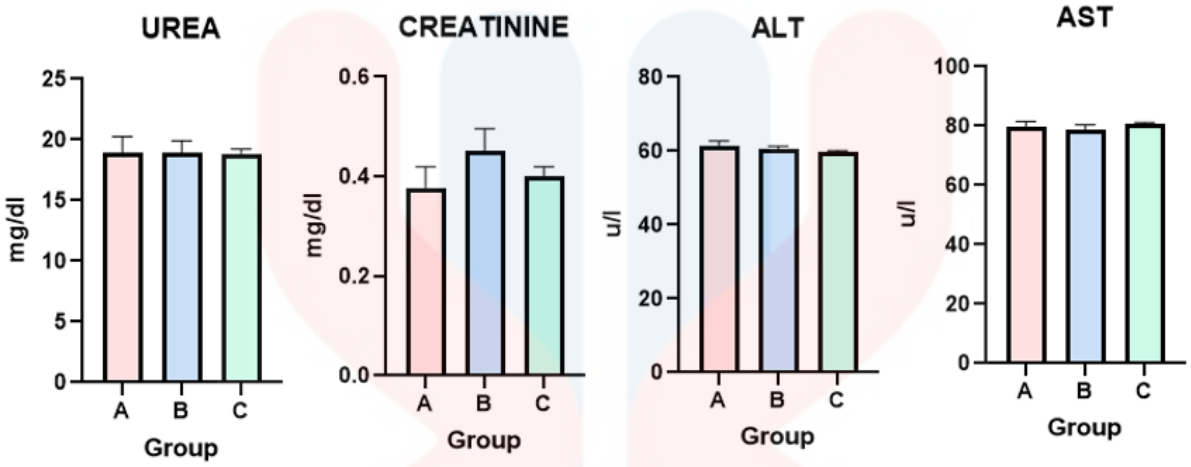


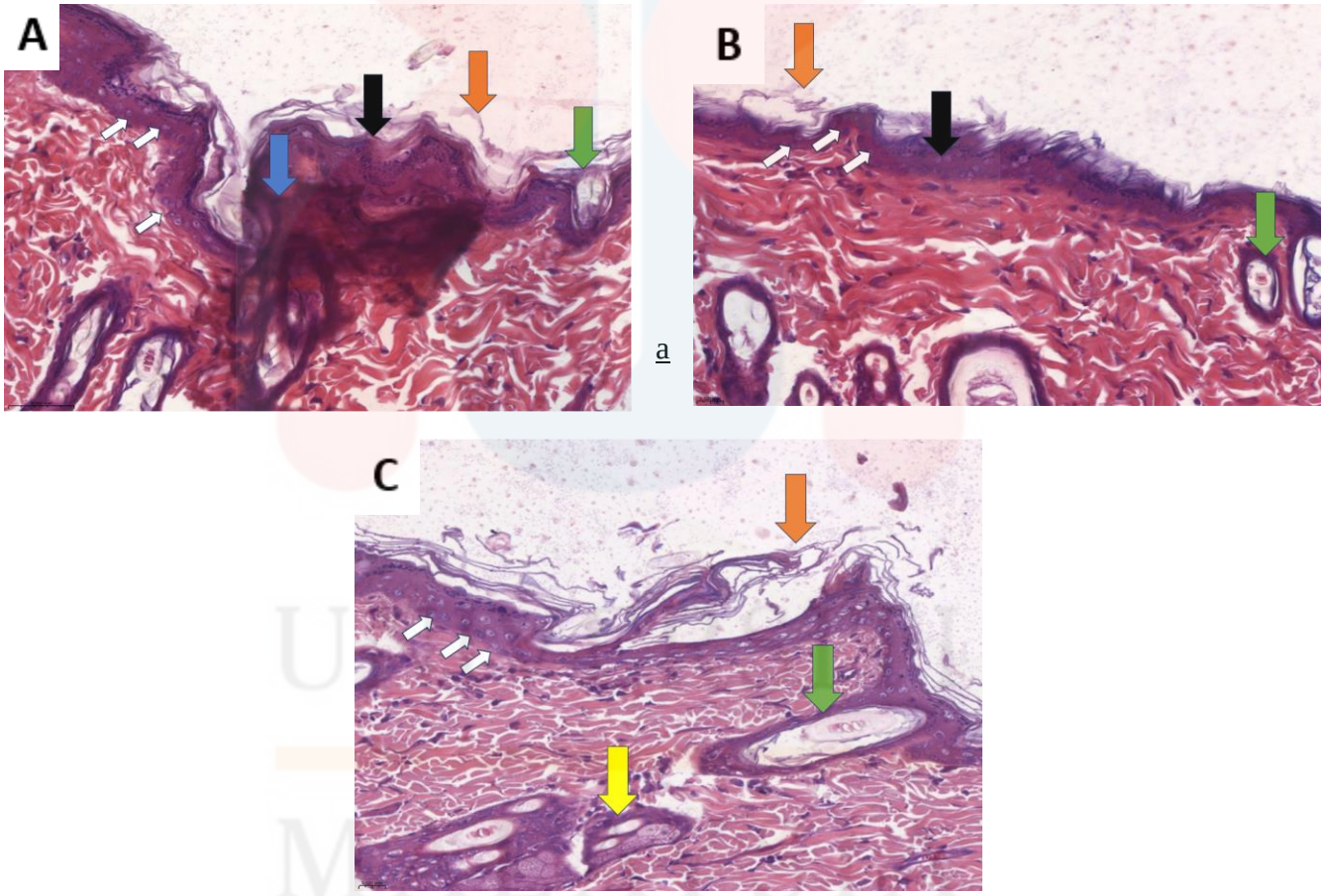
Figure 6: The serum panel data shown as mean and ± SEM. *p < 0.05, and ns: p > 0.05)

Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test for normally distributed data and Kruskal Wallis followed by Dunn's multiple comparisons test for non-normally distributed data (ns, p > 0.05; * p < 0.05; ** p < 0.01; *** p < 0.001, and **** p < 0.0001).

Mean Urea serum panel denote no significant differences of urea from the treated group as compared to the other groups. Creatinine value of Group A was significantly lower compared to Group B (M=0.3780, SD=0.04147 vs. M=0.4520, SD= 0.04438, P=0.0346) Mean values between A-C and B-C are not significant with p= 0.7326 and p= 0.2172 respectively. Analysis suggests that the mean ALT between all Groups are not significant. Mean values between A-B, A-C and B-C are not significant with p>0.05. Dunn's multiple comparisons test analysis suggests that the mean AST between all groups are not significant. Mean values between A-B, A-C and B-C are not significant

4.3 HISTOLOGICAL ANALYSIS

Every mammal's skin has a similar, layered structure. The skin's outermost layer, the epidermis, has an ectodermal origin. Additional layers of mesodermal origin include the dermis, subcutaneous adipose tissue, nerves, musculature, and vessels (Niczyporuk, 2018). It is observed that there are some minor pathological changes that occurred to the skin such as hyperkeratosis and detachment of the keratin layer. There are no cells signifying inflammatory changes have occurred.



a

Figure 7: Histopathology of Skin

The Photomicrograph of the different treated groups showing clean intact architecture of the skin. (A) 3% C+PVA, (B) 1.5% C+PVA+ SA and (C) 3% C+PVA+ SA stained with H&E, under 40x magnification. White arrow the presence of keratinocytes, Blue Arrow the hyperkeratosis present in (A), Black Arrow thickening of Stratum Granulosum, Orange arrow loose/detached keratin layer, Green arrow hair follicle, Yellow arrow sebaceous gland.

The liver's normal microscopic structure is made up of acini and hexagonal lobules. The portal

triad, which consists of the bile duct (BD), hepatic artery (HA), and portal vein (PV), is located in hexagonal lobules that are centred on the central vein (CV) (Palipoch *et al.*, 2013). There are no significant findings that support pathological changes in the tissue. Presence of haemorrhage is expected due to post mortem changes after CO2 euthanasia.

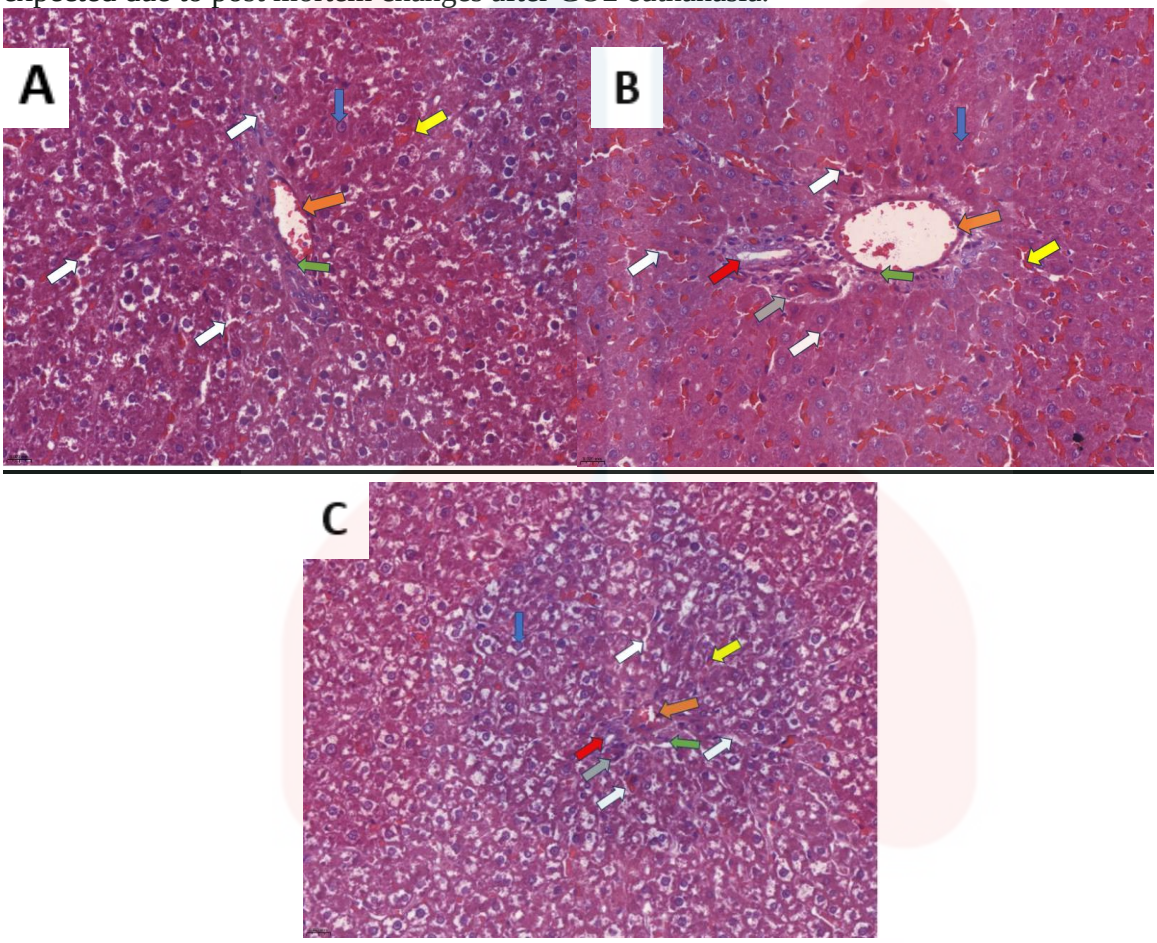


Figure 8: Histopathology of Liver

The Photomicrograph of the different treated groups showing clean intact architecture of the liver. (A) 3% C+PVA, (B) 1.5% C+PVA+ SA and (C) 3% C+PVA+ SA stained with H&E, under 40x magnification. White arrow shows sinusoidal spaces, Blue Arrow shows hepatocyte, Yellow Arrow shows RBC in sinusoid, Orange arrow shows the central vein, Green arrow shows the Kupffer cell, Grey arrow shows the bile duct and Red arrow shows the hepatic artery.

Rat kidney histology typically consists of blood vessels, glomeruli, tubules, and interstitium (Palipoch *et al.*, 2013). There are no significant findings that support pathological changes in the tissue. The normal structures appear intact. Presence of haemorrhage is expected due to post mortem changes after CO2 euthanasia.

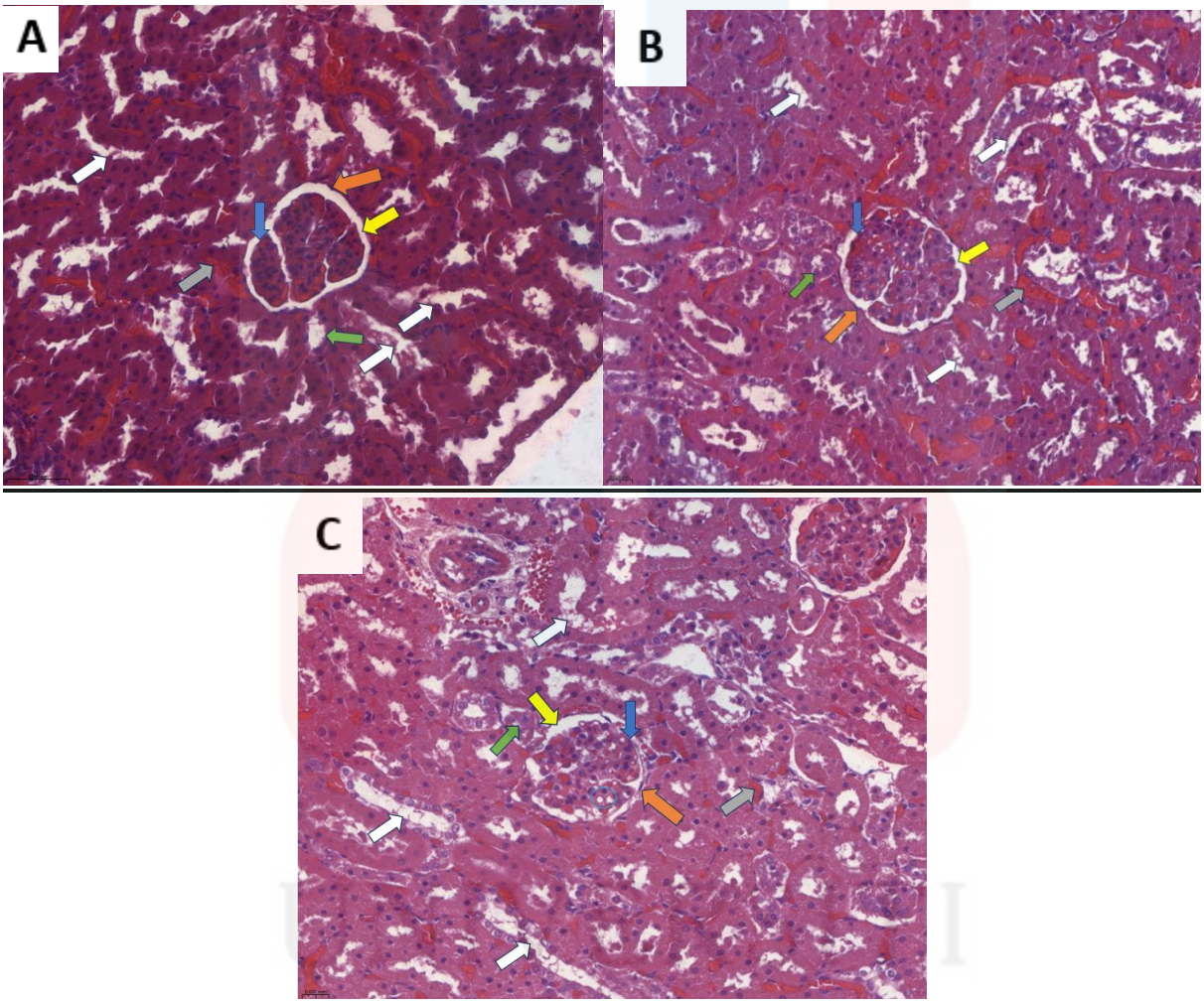


Figure 9: Histopathology of Kidney

The Photomicrograph of the different treated groups showing architecture of the kidney glomerulus. (A) 3% C+PVA, (B) 1.5% C+PVA+ SA and (C) 3% C+PVA+ SA stained with H&E, under 40x magnification. White arrow shows the proximal convoluted tubules, Blue Arrow shows the glomerular capsule, Yellow arrow shows the Bowman's Capsule, Orange arrow shows the podocyte, Green arrow shows the distal convoluted tubule, Grey arrow shows RBC, Blue Circle shows the capillary lumen of the glomerulus (C).

Histopath Statistical Analysis

All samples were scored 1 since there was minimal presence of Inflammatory cells present in the Skin, Kidney and Liver tissues. No statistical tests are needed since the scoring across all samples and groups were the same. 1 sample from Group C was excluded for scoring due to poor histological sample. Further criteria of scoring is suggested to comprehensively analyse the histopathological samples.

5.0 DISCUSSION

Gynura procumbens has garnered significant attention in Malaysia due to its potential pharmaceutical properties. A growing body of research is being undertaken to explore its therapeutic applications, aiming to enhance the quality of life for both humans and animals. Comparative analyses of histopathological and biochemical data from studies on *G. procumbens*, particularly those involving animal models assessed for acute dermal toxicity, have yielded consistent and reliable results.

The experiment uses a safe and appropriate dose of compounds for the construction of biofilms utilized in the study, while also providing insight into the dose-response relationship. Acute dermal toxicity (ADT) is commonly employed as a preliminary assessment before conducting more extensive toxicity studies on novel compounds. The behaviours of the animals had no significant changes, however there was an increased grooming by the rats 12 hours after removal of biofilm. This is expected to be due to physical changes that were performed on the rats from application and removal of the biofilms.

The Urea and AST concentrations have been analysed to determine no significant changes in between the 3 biofilm groups. Creatinine and ALT values between group A-B was significant. Increased muscle metabolism brought on by physical activity or movement can raise the production and excretion of ALT, an enzyme mainly present in muscle and liver cells, as well as creatinine, a byproduct of muscle creatine metabolism. The increased muscle activity during the rats' physical activities may cause these biomarkers' levels to vary, which could lead to discrepancies in the data. (Amin *et al*., 2021; Nathwani *et al*., 2005) The mean serum biochemistry values of groups treated with the biofilms are well within the normal reference range. This is

considered to not contribute to any deleterious effect towards the SDR in any systemic capacity. Priority is directed towards the study of serum biochemistry panels of the kidney and liver due to their main function to filter toxins that are present in the blood. (Reduan *et al*., 2020) Additionally if there is no constraint of model, Complete Blood Count will also be advised to be conducted as blood is the major transport system that's present in the body. Toxins that exist will directly affect the various cells present in the blood circulation which will be reflected in the hematological panel that will be useful for gauging the effects of toxins that's present systemically. (Reduan *et al*., 2020)

Following biochemistry analyses and observations, it is typically crucial to look at the histopathologic results of the target organs or the organ being studied in toxicity studies in order to draw a more accurate conclusion and assess the chemical compound being studied. (Hothorn & Hajian, 1999) When observing the histopathology of the samples, there are no significant changes in the tissue samples that were collected. There was no loss of epidermis layer in all the samples, no presence of hemorrhage or congestion in blood vessels. Appendages such as hair follicles also showed no to minor changes. The primary concern during the start of the experiment was the possibility of developing dermatitis in the experiment, however there was no inflammatory cells found in the skin, further study is needed to determine if the GP biofilms produced exhibit some anti inflammatory and antibacterial properties that could have prevented infiltration of leukocytes. However a total of 4 samples contained the separation of the epidermis, dermis and/or subcutaneous layer. This is believed to be due to poor processing of the sample for histopathology. The findings in liver and kidney histopathology were normal without any lesions, signifying the consistency in the findings between the mean serum biochemistry panels of both organs and histopathology.

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATIONS

In conclusion, for Acute Dermal Toxicity (ADT) all three biofilms showed minor erythema with no edema based on the Draize Dermal Irritation Scoring which resolved without intervention in 2-6 days after administering the biofilm. There were no long term behavioural changes that were observed in the animals before and after induction of the biopolymer films. The serum biochemistry parameters from Sprague Dawley Rats (SDR) across all three groups used for ADT testing showed mean normal biochemistry parameters within the reference range. A significant difference was observed in mean Creatinine and ALT readings between Group A (3% C+PVA) and Group B (1.5% C+PVA+ SA). Histopathology showed normal structure of skin, kidney and liver tissues in all samples. Analysis for Inflammatory Cell Score was not needed due to all samples scoring 1 (absence of inflammatory cells). The research hypothesis is accepted.

A recommendation is to increase the findings' generality and robustness, future research must think about using more samples. By lowering the possibility of errors and maintaining that the findings are more representative of the general sample population, a larger sample size strengthens a study's statistical power.

Another recommendation is, to improve the precision and depth of tissue analysis, an expanded variety of scoring criteria should be used in future research involving histopathology samples. Due to the subjective nature of histopathological evaluation, the full range of cellular and tissue-level variations may not be captured by the application of a single scoring criteria that are essential for a precise diagnosis. Widening the scoring criteria can make it easier to find pathological features or biomarkers that were previously missed.

CHAPTER 7

7.0 REFERENCES

1. Tottoli, E. M., Dorati, R., Genta, I., Chiesa, E., Pisani, S., & Conti, B. (2020). Skin Wound Healing Process and New Emerging Technologies for Skin Wound Care and Regeneration. Pharmaceutics, 12(8), 735. https://doi.org/10.3390/pharmaceutics12080735
2. Abdo, Joe & Sopko, Nikolai & Milner, Stephen. (2020). The applied anatomy of human skin: A model for regeneration. Wound Medicine. 28. 100179. 10.1016/j.wndm.2020.100179.
3. Delwatta, S. L., Gunatilake, M., Baumans, V., Seneviratne, M. D., Dissanayaka, M. L. B., Batagoda, S. S., Udagedara, A. H., & Walpola, P. B. (2018). Reference values for selected hematological, biochemical and physiological parameters of Sprague-Dawley rats at the Animal House, Faculty of Medicine, University of Colombo, Sri Lanka. Animal models and experimental medicine, 1(4), 250–254. https://doi.org/10.1002/ame2.12041
4. Girling, S. J., Campbell-Palmer, R., Pizzi, R., Fraser, M. A., Cracknell, J., Arnemo, J., & Rosell, F. (2015). Haematology and Serum Biochemistry Parameters and Variations in the Eurasian Beaver (Castor fiber). PloS one, 10(6), e0128775. https://doi.org/10.1371/journal.pone.0128775
5. Silva-Santana, G., Bax, J. C., Fernandes, D. C. S., Bacellar, D. T. L., Hooper, C., Dias, A. A. S. O., Silva, C. B., de Souza, A. M., Ramos, S., Santos, R. A., Pinto, T. R., Ramão, M. A., & Mattos-Guaraldi, A. L. (2020). Clinical hematological and biochemical parameters in Swiss, BALB/c, C57BL/6 and B6D2F1 Mus musculus. Animal models and experimental medicine, 3(4), 304–315. https://doi.org/10.1002/ame2.12139

6. Gurina TS, Simms L. Histology, Staining. [Updated 2023 May 1]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK557663/

7. Eelen, G., Treps, L., Li, X., & Carmeliet, P. (2020). Basic and Therapeutic Aspects of Angiogenesis Updated. Circulation Research, 127(2), 310–329. https://doi.org/10.1161/circresaha.120.316851

8. Katare, A. K., Singh, B., Kumar, S., Roy, S., Gupta, A. P., Kumar, A., Singh, B., Tabassum, A., & Sharma, A. K. (2022). Optimisation of Extraction Process for Negundoside and Agnuside from Vitex Negundo L. Leaves Using Soxhlet Extraction, HPLC–MS/MS, and CCD-RSM Methods. *Chemistry Africa*, *5*(4), 907–915. https://doi.org/10.1007/s42250-022-00383-8

9. Brumberg, V., Astrelina, T., Malivanova, T., & Samoilov, A. (2021). Modern Wound Dressings: Hydrogel Dressings. Biomedicines, 9(9), 1235. https://doi.org/10.3390/biomedicines9091235

10. Jobaer, M. A., Ashrafi, S., Ahsan, M., Hasan, C. M., Rashid, M. A., Islam, S. N., & Masud, M. M. (2023). Phytochemical and Biological Investigation of an Indigenous Plant of Bangladesh, Gynura procumbens (Lour.) Merr.: Drug Discovery from Nature. Molecules (Basel, Switzerland), 28(10), 4186. https://doi.org/10.3390/molecules28104186

11. Kim, H. H., Ha, S. E., Vetrivel, P., Bhosale, P. B., Kim, S. M., & Kim, G. S. (2021). Potential Antioxidant and Anti-Inflammatory Function of Gynura procumbens Polyphenols Ligand. International journal of molecular sciences, 22(16), 8716. https://doi.org/10.3390/ijms22168716

12. Mou, Kanzil & Dash, Pritesh. (2016). A COMPREHENSIVE REVIEW ON GYNURA PROCUMBENS LEAVES. International Journal of Pharmacognosy. 3. 167-174. 10.13040/IJPSR.0975-8232.IJP.3(4).167-174.
13. Nagle SM, Stevens KA, Wilbraham SC. Wound Assessment. [Updated 2023 Jun 26]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK482198/
14. Negut, I., Grumezescu, V., & Grumezescu, A. M. (2018). Treatment Strategies for Infected Wounds. Molecules (Basel, Switzerland), 23(9), 2392. https://doi.org/10.3390/molecules23092392
15. Sutthammikorn, N., Supajatura, V., Yue, H., Takahashi, M., Chansakaow, S., Nakano, N., Song, P., Ogawa, T., Ikeda, S., Okumura, K., Ogawa, H., & Niyonsaba, F. (2021). Topical Gynura procumbens as a Novel Therapeutic Improves Wound Healing in Diabetic Mice. Plants (Basel, Switzerland), 10(6), 1122. https://doi.org/10.3390/plants10061122
16. Ahmad Nazri, K. A., Haji Mohd Saad, Q., Mohd Fauzi, N., Buang, F., Jantan, I., & Jubri, Z. (2021). Gynura procumbens ethanol extract improves vascular dysfunction by suppressing inflammation in postmenopausal rats fed a high-fat diet. Pharmaceutical biology, 59(1), 1203–1215. https://doi.org/10.1080/13880209.2021.1970199
17. Chopra, H., Bibi, S., Kumar, S., Khan, M. S., Kumar, P., & Singh, I. (2022). Preparation and Evaluation of Chitosan/PVA Based Hydrogel Films Loaded with Honey for Wound Healing Application. Gels, 8(2), 111. https://doi.org/10.3390/gels8020111
18. OECD (2017), Test No. 402: Acute Dermal Toxicity, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264070585-en.

19. Ediyilyam, S., George, B., Shankar, S. S., Dennis, T. T., Wacławek, S., Černík, M., & Padil, V. V. T. (2021). Chitosan/Gelatin/Silver Nanoparticles Composites Films for Biodegradable Food Packaging Applications. Polymers, 13(11), 1680. https://doi.org/10.3390/polym13111680
20. Charan, J., & Kantharia, N. D. (2013). How to calculate sample size in animal studies?. Journal of pharmacology & pharmacotherapeutics, 4(4), 303–306. https://doi.org/10.4103/0976-500X.119726
21. Alturkistani HA, Tashkandi FM, Mohammedsaleh ZM. Histological Stains: A Literature Review and Case Study. Glob J Health Sci. 2015 Jun 25;8(3):72-9.
22. https://animal.research.uiowa.edu/iacuc-guidelines-anesthesia
23. Abou-Ismail, U. A., Burman, O. H., Nicol, C. J., & Mendl, M. (2007). Let sleeping rats lie: Does the timing of husbandry procedures affect laboratory rat behaviour, physiology and welfare? *Applied Animal Behaviour Science*, *111*(3–4), 329–341. https://doi.org/10.1016/j.applanim.2007.06.019
24. Reduan, F. H., Shaari, R. M., Sayuti, N. S. A., Mustapha, N. M., Bakar, M. Z. A., Sithambaram, S., & Hamzah, H. (2020). Acute and subacute dermal toxicity of ethanolic extract of Melastoma malabathricum leaves in Sprague-Dawley rats. *Toxicological Research*, *36*(3), 203–210. https://doi.org/10.1007/s43188-019-00013-5
25. Ankomah, A. D., Boakye, Y. D., Agana, T. A., Boamah, V. E., Ossei, P. P. S., Adu, F., & Agyare, C. (2022). Evaluation of Dermal Toxicity and Wound Healing Activity of Cnestis ferruginea Vahl ex DC. *Advances in Pharmacological and Pharmaceutical Sciences*, *2022*, 1–11. https://doi.org/10.1155/2022/5268613

26. Chuong, C. M., Nickoloff, B. J., Elias, P. M., Goldsmith, L. A., Macher, E., Maderson, P. A., Sundberg, J. P., Tagami, H., Plonka, P. M., Thestrup-Pederson, K., Bernard, B. A., Schröder, J. M., Dotto, P., Chang, C. M., Williams, M. L., Feingold, K. R., King, L. E., Kligman, A. M., Rees, J. L., & Christophers, E. (2002). What is the 'true' function of skin?. Experimental dermatology, 11(2), 159–187. https://doi.org/10.1034/j.1600-0625.2002.00112.x

27. Shi, C., Wang, C., Liu, H., Li, Q., Li, R., Zhang, Y., Liu, Y., Shao, Y., & Wang, J. (2020). Selection of Appropriate Wound Dressing for Various Wounds. *Frontiers in Bioengineering and Biotechnology*, *8*. https://doi.org/10.3389/fbioe.2020.00182

28. Gupta, A., Kowalczuk, M., Heaselgrave, W., Britland, S. T., Martin, C., & Radecka, I. (2018). The production and application of hydrogels for wound management: A review. *European Polymer Journal*, *111*, 134–151. https://doi.org/10.1016/j.eurpolymj.2018.12.019

29. Han, X., Ju, L. S., & Irudayaraj, J. (2023). Oxygenated Wound Dressings for Hypoxia Mitigation and Enhanced Wound Healing. *Molecular Pharmaceutics*, *20*(7), 3338–3355. https://doi.org/10.1021/acs.molpharmaceut.3c00352

30. The 1988 Medical Waste Tracking Act

31. Bolan, S., Padhye, L. P., Kumar, M., Antoniadis, V., Sridharan, S., Tang, Y., Singh, N., Hewawasam, C., Vithanage, M., Singh, L., Rinklebe, J., Song, H., Siddique, K. H., Kirkham, M., Wang, H., & Bolan, N. (2023). Review on distribution, fate, and management of potentially toxic elements in incinerated medical wastes. *Environmental Pollution*, *321*, 121080. https://doi.org/10.1016/j.envpol.2023.121080

32. Alanazi, H. H., Elasbali, A. M., Alanazi, M. K., & Azab, E. F. E. (2023). Medicinal Herbs: Promising Immunomodulators for the Treatment of Infectious Diseases. *Molecules, 28*(24), 8045. https://doi.org/10.3390/molecules28248045

33. Bowler, P. G., Duerden, B. I., & Armstrong, D. G. (2001). Wound Microbiology and Associated Approaches to Wound Management. *Clinical Microbiology Reviews*, *14*(2), 244–269. https://doi.org/10.1128/cmr.14.2.244-269.2001

34. Wernick B, Nahirniak P, Stawicki SP. Impaired Wound Healing. [Updated 2023 Aug 28]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK482254/

35. Percival, S. L., Emanuel, C., Cutting, K. F., & Williams, D. W. (2011). Microbiology of the skin and the role of biofilms in infection. *International Wound Journal*, *9*(1), 14–32. https://doi.org/10.1111/j.1742-481x.2011.00836.x

36. Tan, H., Chan, K., Pusparajah, P., Lee, L., & Goh, B. (2016). Gynura procumbens: An Overview of the Biological Activities. *Frontiers in Pharmacology*, *7*. https://doi.org/10.3389/fphar.2016.00052

37. Simon, L. S. (1999). Role and regulation of cyclooxygenase-2 during inflammation. *The American Journal of Medicine*, *106*(5), 37S-42S. https://doi.org/10.1016/s0002-9343(99)00115-1

38. Ricciotti, E., & FitzGerald, G. A. (2011). Prostaglandins and Inflammation. *Arteriosclerosis Thrombosis and Vascular Biology*, *31*(5), 986–1000. https://doi.org/10.1161/atvbaha.110.207449

39. Qureshi O, Dua A. COX Inhibitors. [Updated 2024 Feb 28]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK549795/

40. Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D., & Bitto, A. (2017). Oxidative Stress: Harms and Benefits for Human Health. *Oxidative Medicine and Cellular Longevity*, *2017*(1). https://doi.org/10.1155/2017/8416763

41. Wallace HA, Basehore BM, Zito PM. Wound Healing Phases. [Updated 2023 Jun 12]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK470443/

42. Sharkawy, A., Barreiro, M. F., & Rodrigues, A. E. (2020). Chitosan-based Pickering emulsions and their applications: A review. *Carbohydrate Polymers*, *250*, 116885. https://doi.org/10.1016/j.carbpol.2020.116885

43. Morin-Crini, N., Lichtfouse, E., Torri, G., & Crini, G. (2019). Applications of chitosan in food, pharmaceuticals, medicine, cosmetics, agriculture, textiles, pulp and paper, biotechnology, and environmental chemistry. *Environmental Chemistry Letters*, *17*(4), 1667–1692. https://doi.org/10.1007/s10311-019-00904-x

44. Alven, S., & Aderibigbe, B. A. (2021). Fabrication of Hybrid Nanofibers from Biopolymers and Poly (Vinyl Alcohol)/Poly (ε-Caprolactone) for Wound Dressing Applications. *Polymers*, *13*(13), 2104. https://doi.org/10.3390/polym13132104

45. Liu, L., Liang, W., Zhang, Y., & Fu, Q. (2023). Nanoencapsulation in polymeric materials: Weaving magical coats for microorganisms. *Nano Today*, *52*, 101973. https://doi.org/10.1016/j.nantod.2023.101973

46. Ottenbrite, R., & Javan, R. (2005). Biological Structures. In *Elsevier eBooks* (pp. 99–108). https://doi.org/10.1016/b0-12-369401-9/00698-7

47. Yannas IV, `Classes of materials used in medicine: natural materials', in: Biomaterials Science ± An introduction to materials in medicine, Ratner BD, Hoffman AS, Schoen FJ, Lemons J (eds), California, Elsevier Academic Press (2004).

48. Nair, L. S., & Laurencin, C. T. (2007). Biodegradable polymers as biomaterials. *Progress in Polymer Science*, *32*(8–9), 762–798. https://doi.org/10.1016/j.progpolymsci.2007.05.017

49. Arif, Z. U., Khalid, M. Y., Sheikh, M. F., Zolfagharian, A., & Bodaghi, M. (2022). Biopolymeric sustainable materials and their emerging applications. *Journal of Environmental Chemical Engineering*, *10*(4), 108159. https://doi.org/10.1016/j.jece.2022.108159

50. Thang, N. H., Chien, T. B., & Cuong, D. X. (2023). Polymer-Based Hydrogels Applied in Drug Delivery: An Overview. Gels, 9(7), 523. https://doi.org/10.3390/gels9070523

51. Homayun, B., Lin, X., & Choi, H. J. (2019). Challenges and Recent Progress in Oral Drug Delivery Systems for Biopharmaceuticals. Pharmaceutics, 11(3), 129. https://doi.org/10.3390/pharmaceutics11030129

52. Badilli, U., Gumustas, M., Uslu, B., & Ozkan, S. A. (2018). Lipid-based nanoparticles for dermal drug delivery. In *Elsevier eBooks* (pp. 369–413). https://doi.org/10.1016/b978-0-12-813663-8.00009-9

53. Adepu, S., & Ramakrishna, S. (2021). Controlled Drug Delivery Systems: Current Status and Future Directions. *Molecules*, *26*(19), 5905. https://doi.org/10.3390/molecules26195905

54. Waghulde, S. (2013). Development, Recent Inventions and Evaluation Techniques of Transdermal Drug Delivery System - A Review. International Journal of Pharmaceutical and Phytopharmacological Research, 3.

55. Shamsuddin, N. M., Yusup, S., Ibrahim, W. A., Bokhari, A., & Chuah, N. L. F. (2015). Oil extraction from Calophyllum inophyllum L. via Soxhlet extraction: Optimization using response surface methodology (RSM). *2022 13th Asian Control Conference (ASCC)*, 1–6. https://doi.org/10.1109/ascc.2015.7244791

56. Laaldin, N., Baloch, S. R., Noor, A., Aziz, A., Gul, A., Rajput, T. A., & Babar, M. M. (2019). Animal models: bridging cross-species variation through animal biotechnology. In *Elsevier eBooks* (pp. 183–207). https://doi.org/10.1016/b978-0-12-816352-8.00008-4

57. Qureshi, D., Sahoo, A., Mohanty, B., Anis, A., Kulikouskaya, V., Hileuskaya, K., Agabekov, V., Sarkar, P., Ray, S. S., Maji, S., & Pal, K. (2021). Fabrication and Characterization of Poly (vinyl alcohol) and Chitosan Oligosaccharide-Based Blend Films. *Gels*, *7*(2), 55. https://doi.org/10.3390/gels7020055

58. Farazin, A., Mohammadimehr, M., Ghasemi, A. H., & Naeimi, H. (2021). Design, preparation, and characterization of CS/PVA/SA hydrogels modified with mesoporous Ag2O/SiO2 and curcumin nanoparticles for green, biocompatible, and antibacterial biopolymer film. *RSC Advances*, *11*(52), 32775–32791. https://doi.org/10.1039/d1ra05153a

59. Mazlan N.H., Lopez-Salesanksy N., Burn C.C., Wells D.J. Mouse identification methods and potential welfare issues: A survey of current practice in the UK. Anim. Technol. Welf. 2014;13:1–10.

60. Hinchcliffe, J. K., Mendl, M., & Robinson, E. S. J. (2020). Investigating hormone-induced changes in affective state using the affective bias test in male and female rats. Psychoneuroendocrinology, 115, 104647. https://doi.org/10.1016/j.psyneuen.2020.104647

61. Du Sert, N. P., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S. T., Howells, D. W., Hurst, V., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., . . . Würbel, H. (2020). Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0. *PLoS Biology*, *18*(7), e3000411. https://doi.org/10.1371/journal.pbio.3000411

62. Hemmati, M., Ghasemzadeh, A., Malek-Kheili, M. H., Khoshnevisan, K., & Koohi, M. K. (2016). Investigation of acute dermal irritation/corrosion, acute inhalation toxicity and cytotoxicity tests for Nanobiocide®. *ResearchGate*, *1*(1), 23–29. https://doi.org/10.7508/nmrj.2016.01.004

63. Applied Biological Materials Inc, Cat. No. T0103, T0104

64. Son, I., Lee, S., Kim, D., Jeong, H., Cho, S. H., Lee, E. Y., Lee, S. D., Ahn, S. H., & Kim, S. (2014). A Pilot Study on Single-dose Toxicity Testing of Scolopendrid Pharmacopuncture in Sprague-Dawley Rats. Journal of pharmacopuncture, 17(2), 57–66. https://doi.org/10.3831/KPI.2014.17.017

65. Palipoch, S., & Punsawad, C. (2013). Biochemical and histological study of rat liver and kidney injury induced by Cisplatin. Journal of toxicologic pathology, 26(3), 293–299. https://doi.org/10.1293/tox.26.293

66. Niczyporuk, M. (2018). Rat skin as an experimental model in medicine. *Progress in Health Sciences*, *8*(2), 223–228. https://doi.org/10.5604/01.3001.0012.8351

67. Gibson-Corley, K. N., Olivier, A. K., & Meyerholz, D. K. (2013). Principles for valid histopathologic scoring in research. Veterinary pathology, 50(6), 1007–1015. https://doi.org/10.1177/0300985813485099

68. Amin, R., Ahn, S., & Moudgil, A. (2021). Kidney and urinary tract disorders. In *Elsevier eBooks* (pp. 167–228). https://doi.org/10.1016/b978-0-12-817962-8.00010-x

69. Nathwani, R. A., Pais, S., Reynolds, T. B., & Kaplowitz, N. (2005). Serum alanine aminotransferase in skeletal muscle diseases. Hepatology (Baltimore, Md.), 41(2), 380–382. https://doi.org/10.1002/hep.20548

70. Hothorn, L. A., & Hajian, G. (1999). Biostatistics in Toxicology. In *Elsevier eBooks* (pp. 25–41). https://doi.org/10.1016/b978-012473270-4/50061-4

CHAPTER 8

8.0 APPENDIX

Figure 10: Equipment preparation

Figure 11: SDR after induction of biofilms

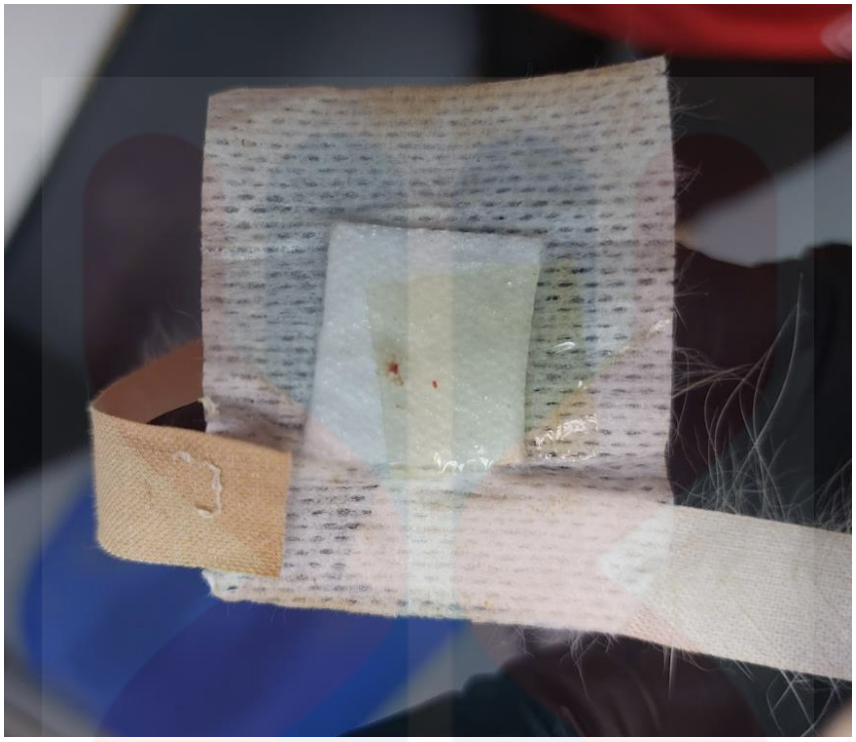


Figure 12: Biofilm removal post 24 hours

Figure 13: Euthanasia of rats using Carbon Dioxide gas

Figure 14: Cardiac phlebotomy

Figure 15: Skin sample collection for Histopathology

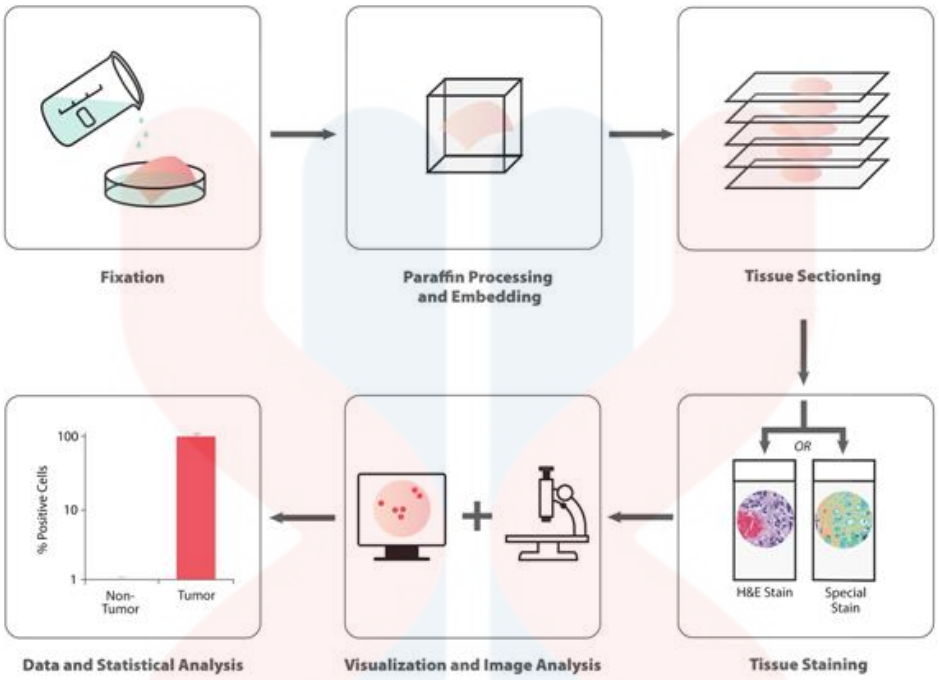


Figure 16: Histopathology procedure (Applied Biological Materials Inc., 2024)