ACUTE TOXICITY, ANTIOXIDANT AND WOUND HEALING POTENTIAL OF ETHANOLIC EXTRACT OF *GONIOTHALAMUS UMBROSUS* IN RATS

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DISSERTATION SUBMITTED IN FULFILLMENT OF THE REGUIREMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

INSTITUTE OF BIOLOGICAL SCIENCE
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ABSTRACT

This study was conducted to evaluate the effects of topical application of an ethanol extract of *Goniothalamus umbrosus* on the rate of skin wound healing closure and the histology of healed wound.

The animal groups were topically treated with 0.2 ml of each vehicle (gum acacia), Intrasite gel, 100 and 200 mg/ml of ethanol leaf extract, respectively. An area of uniform wound 2.00 cm in diameter was excised from the nape of the dorsal neck of all rats with the aid of round seal using circular stamp.

Macroscopically, skin wound dressed with rhizomes extract and Intrasite gel showed significantly healed earlier than those treated with vehicle.

Histological analysis of healed skin wounds dressed with *G. umbrosus* ethanol leaf extract showed comparatively at wound closure and healed wound contained less inflammatory cells and more collagen with angiogenesis compared to wounds dressed with vehicle.

In conclusion, skin wounds dressed with leaf extract showed significantly enhanced the acceleration of wound healing enclosure in rats.

ABSTRAK

Kajian ini dilakukan untuk menilai kesan penggunaan ekstrak pokok *Goniothalamus umbrosus* dalam etanol pada permukaan kulit dengan menggunakan ekstrak etanol daun dalam menilai kadar penutupan penyembuhan luka dan penyembuhan luka secara histologi.

Sebahagian luka berdiameter 2.0 cm yang seragam telah dipotong dan diasingkan daripada bahagian tengkuk leher semua tikus yang digunakan. Kumpulan haiwan dirawat dengan 0.2 ml daripada setiap vektor (gam acacia), gel intrasite, 100 dan 200 mg/ml daun yang diekstrak menggunakan etanol, masing-masing pada permukaan kulitnya. Secara keseluruhannya, luka dirawat dengan ekstrak rizom dan gel intrasite telah sembuh dengan lebih cepat berbanding dengan luka yang dirawat dengan vektor.

Analisis histologi luka yang dirawat dengan ekstrak daun *G. umbrosus* dalam etanol menunjukkan kelebaran pada luka yang lebih rendah dan luka yang sembuh mempunyai sel meradang yang rendah dan mempunyai kolagen dengan angiogenesis berbanding kepada luka yang dirawat dengan vektor.

Kesimpulannya, luka yang dirawat dengan ekstrak daun menunjukkan kadar penyembuhan yang lebih cepat pada tikus.

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LIST OF ABBREVIATIONS

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylene diamine tracetic acid
et al.	And other people
EtOH	Ethanol
FRAP	Ferric Reducing Antioxidant Power
GAG	Glycosaminoglycans
GGT	Gamma-Glutamyl Transferase
GPx	Glutathione Peroxidase Activity
H&E	Hematoxylin and eosin
MDA	Malondialdehyde
MIC	Minimum inhibitory concentration
NADPH	Nicotinamide adenine dinucleotidephosphatehydride
RNS	Reactive nitrogen species
SD	Standard deviation
ROS	Reactive oxygen species
SEM	Standard Eror of The Mean
SOD	Superoxide dismutase
TPC	The total phenolic compounds
VEGF	Vascular endothelial growth factor
UMMC	University Malaya Medical Center

LIST OF SYMBOLS AND UNITS

%	Percentage
+	Plus
_	Minus
×	Multiply
=	Equal to
±	plus minus
<	Less than
/	Divide by
˚C	Degree Celsius
Kg	Kilogram
mg	Milligram
ml	Milliliter
mmol	Millimole
Mm	Millimeter
Min	Minute/s
nm	Nanometer
µM	Micromole
µM	Micrometer
µl	Microliter

LIST OF APPENDIX

CHAPTER ONE

1. INTRODUCTION

Plant extracts have been used as wound healing agents since ancient time (Wang *et al*., 2002; Houghton *et al*., 2005; Nayak *et al*., 2007; Rasal *et al*., 2008; Nayak *et al*., 2009). The usage of traditional medicinal remedies and plants in the treatment of burns and wounds is viewed as an important mode to improve healing and in the same time to reduce the financial burden in the economically deprived societies of the developing world. Several plants and herbs have been used experimentally to treat skin disorders, including wound injuries in traditional medicine like *Rafflesia Hasseltii* (Abdulla *et al*., 2009). *Morinda citrifolia* (Rasal *et al*., 2008), *Momordica charantia* (Sharma *et al*., 2009) and *Limonia acidissima* Linn (Ilango and Chitra, 2010).

A wound can be defined as a disruption of the normal anatomical relationships of tissues as a result of injury. The injury may be intentional such as a surgical incision or accidental following trauma. Enoch and Leaper (2008) defined wound as a break in the epithelial integrity of the skin and may be accompanied by disruption of the structure and function of underlying normal tissue, may also result from a contusion, hematoma, laceration or an abrasion (Enoch *et al*., 2006).

There are several causes or factors, which may interfere with wound healing such as traumatic (mechanical, chemical, physical and surgery), ischemia (e.g. arterial leg ulcer and pressure sore) (Branom and Rappl, 2001). Normal wound healing response begins as soon as the tissue is injured. Wound healing is the process of repair that follows injury to the skin and other soft tissues. Following injury, an inflammatory response occurs and the cells below the dermis begin to increase the collagen production. Later, the epithelial tissue is regenerated (Souba and Wilmore, 1999). It is accepted that wound repair is an immune-mediated physiologic mechanism (Singer and Clark, 1999).

Wound healing or wound repair is an intricate process in which the skin repairs itself after injury (Nguyen *et al*., 2009). Wound infection is most common in developing countries because of poor hygienic conditions (Senthil Kumar *et al.,* 2006), in some part of the world like Malaysia, wounds are mostly due to diabetes. Hence, for infection control, and for the restoration of disrupted anatomical continuity and disturbed functional status of the skin, appropriate method for healing of wounds is essential (Singh *et al*., 2006).

Wound can be classified as acute or chronic and as partial thick or fully thick wounds. Acute wounds are defined as wounds that heal in a predictable and expected period of time. Chronic wounds are usually occurs in compromised patients who have an underlying pathology such as poor circulation or diabetes. Partial thickness wounds involve the epidermis and may or may not involve the dermis. These wounds are shallow, moist and painful. They mostly heal first with the initial inflammation response, then re-epithelialization. However, in full thickness wounds, healing begins after injury initiates a series of cellular and biochemical events that occur in coordinated and overlapping phases in the healthy host, which results in healing (Branom and Rappl, 2001).

Healing of wounds starts from the moment of any physical hurt caused by cutting, shooting, etc., and can continue for varying periods of time depending on the extent of wounding. The process can be broadly categorized into three stages; inflammatory phase, proliferate phase, and finally the remodeling phase which ultimately determines the strength and appearance of the healed tissue (Sumitra *et al*., 2005).

Wound healing is a complex series of interrelated events that are mediated through the phases by a wide range of chemically co-ordinate cellular processes as well as hormonal influences. Chemical mediators such as growth factors, cytokines (small protein hormones that influence normal cell function) and chemokines (chemical mediators

with a powerful chemotactic ability) are combined to co-ordinate the healing process, consequently return the damage tissue to as near normal function as possible, thereby allowing the body to repair injured tissue and restore skin integrity (Chan *et al*., 2008).

The process highly required the coordinated integration of cellular, physiological, biochemical events and molecular process that ultimately result in connective tissue repair and the formation of fibrous scar. The mechanism and speed of healing, and the eventual nature of the regenerated tissue, depend on the type of wound. However, the process depends on the type of tissues which have been damaged and the nature of the tissue disruption. In wound healing, the wound environment changes with the changing individual health status (Hardy, 1989).

Wound healing is a dynamic process involving cellular, molecular, biochemical and physiological phenomena which result in connective tissue repair and fibrous scar formation and lead to the restoration of anatomical continuity and functional status of the skin (Bowler, 2002).

The wound-healing process consists of four highly integrated and overlapping phases: hemostasis, inflammation and tissue remodeling or resolution (Gosain and DiPietro, 2004). These phases and their biophysiological functions must occur in the proper sequence, at a specific time, and continue for a specific duration at an optimal intensity (Mathieu *et al*., 2006). There are many factors that can affect wound healing which interfere with one or more phases in this process, thus causing improper or impaired tissue repair.

1.1 Study objective

1.1.1 General objective

The general objective of this study is to evaluate the potential of ethanol extract of *G. umbrosus* leaves in accelerating wound healing process in experimentally wound-induced rats.

1.1.2 Specific objective

Through the experimental process, the specific objectives were:

- To determine *in vivo* toxicity of *G. umbrosus* leaf extract.

- To evaluate the wound healing potential of *G. umbrosus* leaf extract.

- To evaluate the wound healing potential of *G. umbrosus* leaf grossly and histology.

<div align="center">**CHAPTER TWO**</div>

2. LITERATURE REVIEW

2.1 Wound healing promoters

Research on wound healing promoters is one of the developing areas in modern biomedical sciences. Many traditional practitioners across the world particularly in countries like Malaysia, Saudi Arabia, India, Africa and China with age old traditional practices have valuable information of many wild plants used for treating wounds and burns.

Kumar (2007) has reported the use of several drugs from plant, mineral and animal origins like Ayurveda for their healing properties under the term '*Vranaropaka'*. Usage of a number of plants and animal products for treatment of cuts, wounds and burns had been documented in the herbal medicine. Some of these plants have been screened scientifically for the evaluation of their wound healing activity in different pharmacological models and human subjects, but the potential of most of the plants remain unexplored (Kumar *et al*., 2007).

Sasidharan (2010) studied the wound healing activity of leaves of *Elaeis guineensis* by incorporating the methanolic extract in yellow soft paraffin in concentration of 10% (w/w) in wound induce experimental rats. They reported that the extract showed significant activity against *Candida albicans* with a minimum inhibitory concentration (MIC) value of 6.25 mg/mL. The results showed that the *E. guineensis* extract has potent wound healing capacity, as an evident from observed better wound closure, improved tissue regeneration at the wound site, and supporting histopathological parameters pertaining to wound healing (Sasidharan *et al*., 2010). Similar observation was reported by Agarwal (2009) while observing the wound healing activity of plantain banana extracts in rats.

Abdulla (2009) studied the wound healing effect of *Rafflesia hasseltii* leaf extracts in rats. They reported that wounds treated with placebo containing 5%, 10% *R. hasseltii* buds extract or intrasite gel showed markedly less scar width at wound enclosure. The granulating tissue contained markedly more collagen and proliferating fibroblasts, with the absence of inflammatory cells as compared to wounds treated with blank placebo. Based on their findings on increased rate of wound closure and contraction together with the histological findings they concluded that *Rafflesia hasseltii* buds extract is very effective in accelerating the wound healing process.

Ilango and Chitra (2010) studied the wound healing and anti-oxidant activities of methanol extract of *Limonia acidissima* Linn (Rutaceae) fruit pulp in Rats. After treatment of the experimental groups they observed increased wound breaking strength, decreased epithelization period, increased wound contraction, increased granulation tissue weight and hydroxyproline concentration were observed in the various groups, compared with the control group. Also, increased activity of anti-oxidant enzymes and catalase levels, were also reported in extract-treated groups when compared to controls. Wound healing activity was statistically significant ($p < 0.001$) in animals treated with 400 mg / kg of the extract. Based on their observations they concluded that methanol extract of *L. acidissima* possesses significant dose-dependent wound healing and anti-oxidant that supported the traditional claim of this plant as a wound healer.

2.2 Physiology of wound healing

Physiologically wound-healing process consists of four highly integrated and overlapping phases: hemostasis, inflammation, proliferation, and tissue remodeling or resolution (Gosain and DiPietro, 2004).

These phases and their biophysiological functions must occur in the proper sequence, at a specific time, and continue for a specific duration at an optimal intensity (Mathieu *et*

al., 2006). There are many factors that can affect wound healing which interfere with one or more phases in this process, thus causing improper or impaired tissue repair. Several different cell types are involved in the wound-healing process, and the cellular activities of any particular cell type may also vary during different stages of repair. The complexity and coordination of the healing process are major hurdles to therapeutic approaches, since any therapeutic must effectively be sequenced to the appropriate stage (Guo and Dipietro, 2010).

(a) Hemostasis Phase

Hemostasis phase begins immediately after wounding with vascular constriction and fibrin clot formation. The clot and surrounding wound tissue release pro-inflammatory cytokines and growth factors such as transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF). Once bleeding is controlled, inflammatory cells migrate into the wound (chemotaxis) and promote the inflammatory phase (Guo and Dipietro, 2010).

(b) Inflammatory Phase

Inflammation is a normal and necessary prerequisite to healing. This response is a non-specific local reaction to tissue damage and or bacterial invasion aroused by the body's defense mechanisms and is an essential part of the healing process. Tissue damage triggers both the complement and cytokine systems. The complement system consists of plasma proteins, as inactive precursors. Activated precursor cascade effect that leads to the release of histamine from the mast cells and results in vasodilatation and increased capillary permeability. This effect is enhanced by kinin system that activates kinogen to kinin. Kinin attracts neutrophils to the wound, enhance phagocytosis and cause pain by stimulating the sensory nerve ending (Dealey, 1994).

The inflammatory phase which is characterized by the sequential infiltration of neutrophils, macrophages and lymphocytes (Gosain and DiPietro, 2004; Broughton *et al*., 2006; Campos *et al*., 2008). A critical function of neutrophils is the clearance of invading microbes and cellular debris in the wound area, although these cells also produce substances such as proteases and reactive oxygen species (ROS), which cause some additional bystander damage. Macrophages play multiple roles in wound healing. In the early wound, macrophages release cytokines that promote the inflammatory response by recruiting and activating additional leukocytes.

Macrophages are also responsible for inducing and clearing apoptotic cells (including neutrophils), thus paving the way for the resolution of inflammation. As macrophages clear these apoptotic cells, they undergo a phenotypic transition to a reparative state that stimulates keratinocytes, fibroblasts, and angiogenesis to promote tissue regeneration (Meszaros *et al*., 2000; Mosser and Edwards, 2008). In this way, macrophages promote the transition to the proliferative phase of healing-lymphocytes migrate into wounds following the inflammatory cells and macrophages, and peak during the late-proliferative/early-remodeling phase.

The role of T-lymphocytes is not completely understood and is a current area of intensive investigation. In addition, skin gamma-delta T-cells regulate many aspects of wound healing, including maintaining tissue integrity, defending against pathogens, and regulating inflammation. These cells are also called dendritic epidermal T-cells (DETC), due to their unique dendritic morphology. DETC are activated by stressed, damaged, or transformed keratinocytes and produce fibroblast growth factor 7 (FGF-7), keratinocyte growth factors, and insulin-like growth factor-1, to support keratinocyte proliferation and cell survival.

DETC also generate chemokines and cytokines that contribute to the initiation and regulation of the inflammatory response during wound healing. While cross-talk

between skin gamma-delta T-cells and keratinocytes contributes to the maintenance of normal skin and wound healing, mice lacking or defective in skin gamma-delta T-cells show a delay in wound closure and a decrease in the proliferation of keratinocytes at the wound site (Jameson and Havran, 2007; Mills *et al*., 2008).

(c) Proliferative Phase

The proliferative phase generally follows and overlaps with the inflammatory phase, and is characterized by epithelial proliferation and migration over the provisional matrix within the wound (re-epithelialization). In the reparative dermis, fibroblasts and endothelial cells are the most prominent cell types present and support capillary growth, collagen formation, and the formation of granulation tissue at the site of injury. Within the wound bed, fibroblasts produce collagen as well as glycosaminoglycans and proteoglycans, which are major components of the extracellular matrix (ECM). Following robust proliferation and ECM synthesis, wound healing enters the final (Guo and Dipietro, 2010).

Proliferative phase take place when the inflammatory phase completed to rebuild, resurface and impart strength to the wound. Usually occur between days 4-14 after injury. Epithelialization, angiogenesis, granulation, tissue formation, and collagen deposition are among the major steps of this phase (Dealey, 1994). The undamaged epithelial cells within the wound margin start to reproduce within hours after injury. Endothelial cells produce vascular endothelial growth factor VEGF, FGF, and PDGF. Keratinocytes produce TGF-α, TGF-β, and KGF (Chan *et al*., 2008). The process of epithelialization is impaired by the presence of necrotic tissue, or slough, which may need to be actively removed by debridement to promote healing (Hardy., 1989; Dealey., 1994). Epithelialization can be facilitated by maintaining moist dressings, protecting the wound from minor repetitive trauma, avoidance of chemical irritants or infection,

debridement and possibly topical oxygen therapy. The process of contraction is a key phase of wound healing and commences approximately a week after wounding if contraction continues for too long, it can lead to disfigurement and loss of function. Through the process of contraction, the entire wound is pulled together and become shrink. Successful contraction results in a smaller wound to be repaired by scar formation, which gradually flattens to a thin white line (Dealey, 1994).

All these growth factors are needed to continue stimulating wound cellular proliferation, angiogenesis, production of extracellular matrix proteins and glycoproteins at the wound site.

(d) Remodeling phase

The final process in wound healing is maturation of the scar. Clinically this stage is the most important because the main feature of this phase is deposition of collagen in an organized network. For a successful wound healing not only closing the wound with sufficient tensile strength is important but also the ultimate goal normal functioning. Remodeling requires the scar to change to fit the tissue. Wound repair is optimal when this remodeling of scar tissue occurs and less than optimal when it does not occur, it is responsible for the final aggregation, orientation, and arrangement of collagen fibers (Hardy, 1989).

The remodeling of the tissue scar leads to a scar that is an a vascular scar in nature, which therefore appears paler than the surrounding tissue. The scar tissue usually appears flattened compared to normal tissue and it does not contain any sebaceous gland and hair follicle. However, in hypertropic scar there is excess formation of scar tissue which cause the persistent raised of the scar sometimes it may extend beyond the original wound into healthy tissue resulting in a keloid scar (Hardy, 1989).

Remodeling phase, regression of many of the newly formed capillaries occurs, so that vascular density of the wound returns to normal. One critical feature of the remodeling phase is ECM remodeling to an architecture that approaches that of the normal tissue. The wound also undergoes physical contraction throughout the entire wound-healing process, which is believed to be mediated by contractile fibroblasts (myofibroblasts) that appear in the wound (Gosain and DiPietro, 2004; Campos *et al*., 2008).

Epidermal stem cells reside in the bulge area of hair follicles and in the basal layer of the epidermis and give rise to the keratinocytes that migrate and re-epithelialize wounds. Normal skin is also a target organ for BMDCs. Two main stem cell populations are present in the bone marrow: hematopoietic SC (HSC) and mesenchymal SC (MSC). BM-MSCs are able to differentiate into a variety of cell types, including adipocytes, osteoblasts, chondrocytes, fibroblasts, and keratinocytes (Cha and Falanga, 2007; Rea *et al*., 2009).

Endothelial progenitor cells (EPCs) derived from the HSC lineage are key cells that contribute to neovascularization. Both BM-MSCs and EPCs are involved in the cutaneous wound-healing process. Wound-induced hypoxia triggers the mobilization of bone marrow EPCs to the circulation, playing a significant role in the process of neovascularization (Wu *et al*., 2007; Liu and Velazquez, 2008; Rea *et al*., 2009).

2.3 Role of antioxidants in wound healing

Many facets of wound healing under redox control require a delicate balance between oxidative stress and antioxidants. While the normal physiology of wound healing depends on low levels of reactive oxygen species and oxidative stress, an overexposure to oxidative stress leads to impaired wound healing. Antioxidants are postulated to help control wound oxidative stress and thereby accelerate wound healing (Fitzmaurice SD., 2011).

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols (Sies, 1997), antioxidants minimize the oxidation of lipid component in cell membranes or inhibit the volatile organic compounds and the conjugated dine hydroperoxides which are known to be carcinogenic (Singh *et al*., 2006). Shukla (1999) observed in treated wounds, the decreased levels of all antioxidants (enzymatic and nonenzymatic) were restored and increased significantly. These increased *in situ* levels of antioxidant can bring about enhanced healing.

Several studies demonstrated the beneficial effects of antioxidants in accelerating wound healing (Rasal *et al*., 2008; Abdulla *et al*., 2009).Oxidative stress is thought to contribute to development of a wide range of diseases including Alzheimer, diabetes and neurodegeneration (Buijnsters *et al*., 2001).When there is an imbalance between antioxidants and free radicals, oxidative stress will prevail.

The free radicals that damage cellular macromolecules, producing oxidative stress, are scavenged in the human body by a range of antioxidant enzymes and small molecule antioxidants (Buijnsters *et al*., 2001).Hence, Antioxidant activity is popularly used to indicate the ability of an antioxidant to scavenge some radicals. Free radical colorimeter relies on the reaction of an antioxidant with the stable free radical 2, 2-diphenyl-l-picrylhydrazyl (DPPH) dissolved in methanol (Buijnsters *et al*., 2001). DPPH is a relatively stable paramagnetic free radical that accepts electrons or H^+ radicals to become a stable diamagnetic molecule.

2.4 *Goniothalamus umbrosus*

Goniothalamus umbrosus is the second largest genus of *Annonaceae* and widely used in traditional medicinal practices in Asia. *Goniothalamus umbrosus*, or known locally as kenerak, is probably one of the most interesting medicinal plants of the east coast of the Malay Peninsula, grown by generations of traditional practitioners mostly for health-care (Figure 2.1).

Plants are a rich source of secondary metabolites with interesting biological activities. In general, these secondary metabolites are an important source with a variety of structural arrangements and properties (de-Fátima *et al*., 2006). Distinguished examples of these compounds include flavonoids, phenols and phenolic glycosides, saponins and cyanogenic glycosides (Shahidi., 2000 and Shahidi *et al*., 2008). Plants for active compounds have become very significant because these may serve as talented sources of book antibiotic prototypes (Meurer-Grimes *et al*., 1996; Koduru *et al*., 2006). It has been shown that *in vitro* screening methods could provide the needed preliminary observations necessary to select crude plant extracts with potentially useful properties for further chemical and pharmacological investigations (Mathekaga and Meyer, 1998). *Goniothalamus umbrosus* is a plant described firstly by James Sinclair in 1955 (Mat-Salleh *et al*., 2000).

Several studies demonstrated the styrylpyrone goniothalamin, found within the family *Annonaceae*, is one of the bioactive styryl-lactones in the genus *Goniothalamus.* Goniothalamin is the first styryllactone. It was crystallized from the light petroleum extract of *Goniothalamus* speciesbark. It has been shown to possess medicinal properties against various diseases and anti-cancer and apoptosis inducing properties against various human tumour and animal cell lines (Jewers *et al*., 1972; Umar-Tsafe *et al*., 2004). In regards to the pharmacological potentials of *Goniothalamus* species, there is a massive body of evidence to suggest that this taxon has the ability to elaborate series of acetogenins and styryl-lactones which are cytotoxic against a broad array of

cancer cells including breast, colon, kidney and pancreatic carcinoma cells. An exciting fact about the mode of action of styryl-lactones is action via protein kinase (Wiart C, 2007).

This compound can serve as *vitro* (Mosaddik and Haque, 2003), insecticidal (Kabir *et al*., 2003) and anti-fertility agent in rats. In particular, it was reported to exert cytotoxic properties in a variety of tumor cell lines including MCF-7, HeLa cells (Ali *et al*., 1997).

It is demonstrated that goniothalamin induced apoptosis in HL-60 cancer cells, via mitochondrial pathway (Inayat-Hussain *et al*., 2003). This compound has also been reported to be a potent mosquito larvicide in lymphatic filariasis (Kabir *et al*., 2003) and as an antifertility agent in rats without mating or behavioural side effects (Sabourin, 2004).

Figure 2.1: A1 *Goniothalamus umbrosus*, B1 leaves, C1 flower and D1 fruits

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Plant materials

Goniothalamus umbrosus leaf was obtained from Ethno Resources Sdn Bhd, Selangor Malaysia, and identified by comparison with the Voucher specimen deposited at the Herbarium of Rimba Ilmu, University of Malaya, Kuala Lumpur, Malaysia.

3.2 Preparation of plant extract

Goniothalamus umbrosus leaf finely powdered after collected and dried using electrical blender.100 g of fine powder was homogenized in 500 ml of 95% ethanol in conical flask for 3 days. After 3 days the mixture was filtered using a fine muslin cloth followed by filter paper (Whatman No. 1) and concentrated under reduced pressure in an Eyela rotary evaporator (Sigma-Aldrich, USA). The extracts were stored at -20°C.

3.3 Antioxidant assays *in vitro*

The 2, 2-diphenyl-1-picrahydrazyl (DPPH) assays is a method for the analysis of antioxidant activity (Huang *et al*, 2005). This assay is based on the measurements of the reducing ability of antioxidants towards DPPH radical. DPPH is a stable free radical with maximal absorption at 515 nm. It loses this absorption when reduced by an antioxidant or a free radical species (Szabo *et al*., 2007).

The color of the solution changes from a deep purple to yellow when the radical is quenched by antioxidants and the absorbance decreases. The decrease in absorbance at a reaction time is used in determining the antioxidant activity of the tested substances (Szabo *et al*., 2007).The reduction of DPPH by an antioxidant results in the formation of a purple-blue colored solution which gives the measure of the activity by spectrophotometer at 515nm (Buijnsters *et al*., 2001).

3.4 Intrasite gel

Intrasite gel is a trademark for Smith and Nephew Healthcare Limited (Williams, 1994). Intrasite gel is a colorless transparent aqueous gel, which contains 2.3% of a modified carboxymethyl cellulose polymer together with propylene glycol (20%) as a humectants and preservative. When placed in contact with a wound, the dressing absorbs excess exudate and produces a moist environment at the surface of the wound, without causing tissue maceration.

Intrasite may be applied to many different types of wound, including leg ulcers, pressure sores, surgical wounds and extravasation injuries. It is of particular value in the treatment of dry, necrotic wounds, promoting rapid debridement by facilitating rehydration and autolysis of dead tissue. In the management of granulating wounds, Intrasite prevent desiccation, and thus facilitates re-epithelialisation. The gel can form a useful vehicle for the topical application of antimicrobial agents such as metronidazole. Such preparations may be used, in combination with systemic therapy, for the management of wound odor caused by infection with sensitive organisms.

Intrasite gel is an amorphous hydrogel which gently rehydrates necrotic tissue and facilitates autolytic debridement, while being able to loosen and absorb slough and exudates, clearing the way for effective wound healing.

It is also designed for wounds that are granulating and epithelializing. It can also be used to provide the optimum moist wound management environment during the later stages of wound closure. It is non-adherent and does not harm viable tissue or the skin surrounding the wound. This makes the use of Intrasite gel ideal for every stage in the wound management process (Williams, 1994).

3.4.1 Lignocaine HCl (2%, 100 mg/5 ml)

Lignocaine (Delta Veterinary Laboratory PTY LTD, NSW 2011) is a local anesthesia and purchased from experimental animal house, Faculty of Medicine, University Malaya. About one mL of Lignocaine was injected subcutaneous.

3.5 Acute toxicity test
3.5.1 Experimental animals for acute toxicity studies

The acute toxic study was used to determine a safe dose for *G. umbrosus*. Thirty six healthy *Sprague Dawley* rats (18 males and 18 females) were obtained from the Experimental Animal House, Faculty of Medicine, University of Malaya. Each rat weighted between (220 - 250) g. The rats were divided randomly into four groups of night rats each and labeled as vehicle (gum acacia in normal saline); 2 and 5 g/kg of *G. umbrosus* in vehicle, respectively.

The animals were fasted overnight (food but not water) prior dosing. Food was held for a further 3 to 4 h after dosing. The animals were observed for 30 min and 2, 4, 24 and 48h after the administration for the onset of the clinical or toxicological symptoms. Mortality rate was observed over a period of ten days. The animals were sacrificed on day 10 using diethyl ether.

Hematological and histological of liver and kidney parameters were determined following standard methods (Perry, 1980; Yam *et al*., 2008). The study was approved by the ethics Committee for animal experimentation, Faculty of Medicine, University of Malaya. Throughout the experiments, all animals received human care according to the criteria outlined in the "Guide for the Care and Use of laboratory Animals" prepared by the National Academy of Sciences and published by the national Institute of health, Malaysia.

3.6 Wound healing study

3.6.1 Experimental animals for wound healing

In this study, *Sprague Dawley* adult 18 male rats weighing in the range of (200-250g) were purchased from the Animal house, Faculty of Medicine, University of Malaya.

The rats were kept in a specially prepared cages at room temperature (23 - 32°C) with 12 hours light/12 hours dark photoperiod and 50% to 60% humidity in order to maintain normal circadian rhythm in the animal room. The rats were fed *ad labitum* with rodent food pellet and water which was given through special dropper-tipped bottles placed in the cages. The handling of animals is accordance to the experimental protocols which were approved by the Committee for the Supervision of Animal Experimentation, Faculty of Medicine, University of Malaya.

3.6.2 Experimentally induced excision wounds

The animals were anesthetized with 0.09 ml of Ketamine injection (30 mg/kg, 100 mg/ml) and 0.01 ml of Xylazil injection (3 mg/kg, 100 mg/ml) prior to creation of the wounds. The skin shaved by electrical clipper, disinfected with 70% alcohol and injected with one ml of Lignocaine HCl S.C injection (20 mg/ml). An area of uniform wound 2.00 cm in diameter (circular area = 3.14 cm^2) was excised from the nape of the dorsal neck of all rats with the aid of round seal under local and general anesthesia as described previously.

Avoid incision of the muscle layer, and tension of skin was kept constant during the procedure, the entire wound left open, the wound area was measured immediately under by placing a transparent tracing paper over the wound and tracing it out. The tracing paper was placed on1 mm 2 graph sheet, and traced out. The squares were counted and the area was recorded, as described previously (Abdulla *et al*., 2009).

3.6.3 Topical wound application

Wounds of the first group of animals were topically treated with 0.2 ml of vehicle, gum acacia in normal saline (20 mg/ml), twice daily as placebo control group (Shetty *et al*., 2008). Wounds of the second group rats were topically treated with 0.2 ml of Intrasite gel twice daily as a reference standard control. Moreover, 0.2 ml of ethanol extract of *G. umbrosus* in vehicle was applied topically twice daily to the wound of third and fourth groups respectively.

All animals were sacrificed on ten day post-wounding surgery. The wound closure area of each animal was assessed by tracing the wound on days 1, 5, and 10 post-wounding surgery and the wound closure rate was expresses as the percentage of wound area compared with that on post-operative day by using transparency paper and a permanent marker under general anesthesia (a mixture of Ketamine and Xylazil) as described by Nayak and Pinto-Pereira (2006). The wound areas recorded were measured using a graph paper. The percent wounds healing on these days are determined (Chah *et al*., 2006).

3.6.4 Estimation of wound healing (wound closure)

Skin wound areas were traced manually and calculated in square millimeters, the wound was observed daily until complete wound-healing enclosure completely occurs. The wound closure area of each animal was assessed by tracing the wound on days 1, 5 and 10 post-wounding surgery and the wound closure rate was expresses as the percentage of wound area compared with that on post-operative day by using a transparent paper and a permanent marker under general anesthesia (Ketamine and Xylazil) as described previously. The percent of wounds healing on these days were determined (Mahmood *et al*., 2010). Number of days required for falling of scar without any residual raw wound gives the period of epithelization.

3.6.5 Antioxidant enzymes assay in wound homogenate

Granulation tissue was collect in phosphate-buffered saline for the estimation of antioxidant enzymes like superoxide dismutase (Kakkar *et al*., 1984), catalase (Aebi *et al.,* 1974), reduced glutathione (GSH) (Beutler *et al*., 1963) and tissue lipid peroxidation (Konings and Drijiver, 1979).

3.7 Antioxidant measurement from granulation tissue

Wound tissues were collected on day 10 post-wounding from wound tissue area for measurement of antioxidant enzyme activities, glutathion peroxidase and superoxide dismutase. Lipid peroxidation was determined as malondialdehyde of granulation tissue.

3.7.1 Protein determination

The total proteins content of wound tissues were determined from each animal according to the method of Bradford (1976). Briefly, wound tissues collected on day 10 post wounding were homogenized in 1.15% calcium chloride at a ratio of 1:5 (w/v). A 0.1 ml quantity of the homogenate was added to 5 ml of Bradford reagent, mixed, and the absorbance read at 595 nm wavelength against 0.1 M phosphate buffer, pH 7.4. Standards were treated similarly using BSA at concentrations of 0, 20, 40, 60, 80 and 100µg/ml in 0.1 M phosphate buffer at pH 7.4.

3.7.2 Glutathione peroxidase activity (Gpx)

For the determination of GPx activity in the wound tissue, day-10 wound tissues were prepared by homogenizing in 1.15% potassium chloride at a ratio of 1:5 (w/v). They were then centrifuged at 8,000 rpm for 20 min at 4°C. The resultant supernatants were again centrifuged at 35,000 rpm for 1 h at 4°C.

Glutathione peroxidase was determined by the method of Lawrence and Burk (1976). Briefly, the reaction mixture contained 2 ml phosphate buffer (50 m M ; pH 7.0), 0.2 ml

EDTA, 0.3 ml sodium azide (1 m M), 0.1 ml GSH (1 m M) and 0.1 ml NADPH (0.2 m M). 0.2 ml of enzyme solution was added to this mixture and incubated for 5 min at room temperature, and then the reaction started by adding 0.2 ml of 0.25 m M H_2O. Optical density was measured at 340 nm with 20 min intervals. Enzyme activity was measured as micromolar of oxidized NADPH per minute per milligram protein.

3.7.3 Superoxide dismutase activity (SOD)

Homogenized wound tissues were prepared as for GPx on the day-10 post wounding and were analyzed for SOD activity. SOD was determined according to the method of (Beyer and Fridovich 1987). Briefly, the hemolysate was mixed with a substrate mixture consisting of 50 mM phosphate buffer containing 0.1 m M EDTA, pH 7.8, prepared fresh on the day of analysis; L-methionine (0.03g/mL); nitroblue tetrazolium chloride (NBT·2HCl) (1.41 mg/mL); and 1% Triton X-100® (vol/vol). The reaction was started by adding 10 µL riboflavin (4.4mg/100mL). The mixtures were mixed, then put in an aluminum box illuminated with two fluorescent lamps of 20 W for 7 min, and the absorbance was read at 560 nm wavelength. The whole procedure was carried out in a dark place because both of riboflavin and NBT are sensitive to light.

3.7.4 Malondialdehyde (MDA) levels

Malondialdehydelevels in the day-10 wound tissue homogenate were also determined to reflect the level of lipid peroxidation using thiobarbituric acid reactionmethods (Ledwozyw *et al.*, 1986). Briefly, the tissues were homogenized in 1.15% potassium chloride and diluted 1:5 with distilled water before analysis. 2.5 ml of trichloro acetic acid solution was then added the hemolysate and left at room temperature for 15 min. Thiobarbituric acid (1.5 ml) was then added to the mixture,

mixed and the solution incubated in a water bath for 30 min. The tubes were then cooled and shaken vigorously for 30 min. Before adding 4 ml of n-butanol solution. The tubes were then centrifuged by room temperature. The absorbance was read at 595 nm the resulting upper layer was then read using a spectrofluorometer (Shimadzu, Kyoto, Japan).

3.8 Histological processing of healed wounds

At day10 post-surgery, the skin of liver and kidney specimens were collected and fixed in 10% formalin and processed by paraffin tissue processing machine. The tissues were assessed by taking a 5 section, stained with hematoxylin and eosin.

3.9 Statistical analysis

All values were reported as mean ± SEM and the statistical significance of differences among groups were assessed using One-Way Analysis of Variance (ANOVA). The data were considered significant at $p < 0.05$.

3.10 Experimental design

Flow charts demonstrating the step procedures of the experiment.

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│                                                                           │
│   ┌──────────────────────────┐        ┌──────────────────────────┐      │
│   │   Preparation of plant    │        │          Rats             │      │
│   └──────────────────────────┘        └──────────────────────────┘      │
│               │                                    │                      │
│   ┌──────────────────────────┐        ┌──────────────────────────┐      │
│   │ Preparation of the        │        │ 36 rats divided into      │      │
│   │ treatment mixture         │        │ 3 groups                  │      │
│   └──────────────────────────┘        └──────────────────────────┘      │
│               │                                    │                      │
│   ┌──────────────────────────┐        ┌──────────────────────────┐      │
│   │      Toxicity test        │        │ Individually caged, given │      │
│   └──────────────────────────┘        │ standard diet & tap.      │      │
│               │                        └──────────────────────────┘      │
│   ┌──────────────────────────┐                    │                      │
│   │ Antioxidant activity in   │        ┌──────────────────────────┐      │
│   │ vitro                     │        │     Fasted 24 hours       │      │
│   └──────────────────────────┘        └──────────────────────────┘      │
│               │                                    │                      │
│   ┌─────────────────────────────────────────────────────────────┐        │
│   │           Experimentally induced wounds                      │        │
│   └─────────────────────────────────────────────────────────────┘        │
└───────────────────────────────────────────────────────────────────────────┘
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Topical application of vehicles twice a day

G1: were treated twice daily with 0.2 ml 10% Tween 20 as negative control.

G2: were topically dressed with 0.2 ml of commercial Intrasite gel twice daily as reference.

G3&G4: animals were treated topically with 0.2 ml of 100 mg/kg and 200 mg/kg plant extract twice daily, respectively.

Tracing the wound on days 1, 5, 10 post-wounding surgery

Rats sacrificed liver and kidney by diethyl ether; skin wounds healed areas were collected

Macroscopic and Microscope examination

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│                                                         │
│        ┌──────────────────────────────────────┐         │
│        │     Antioxidant of wound homogeneity  │         │
│        └──────────────────────────────────────┘         │
│                           ⇓                             │
│        ┌──────────────────────────────────────┐         │
│        │  Tissue processing of the skin liver  │         │
│        │        and kidney specimens           │         │
│        └──────────────────────────────────────┘         │
│                           ⇓                             │
│        ┌──────────────────────────────────────┐         │
│        │    Stain with Haemotoxylin &Eosin     │         │
│        └──────────────────────────────────────┘         │
│                           ⇓                             │
│        ┌──────────────────────────────────────┐         │
│        │  Histological examination and         │         │
│        │    evaluation by microscopy           │         │
│        └──────────────────────────────────────┘         │
│                                                         │
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CHAPTER FOUR

4. RESULTS

4.1 Acute toxicity

Acute toxicity study the animals were treated with the *G. umbrosus* extracts at a dose of 2 and 5 g/kg were kept under observation for 10 days. All the animals remained alive and did not manifest any significant visible of toxicity at these doses. Thus, clinical observations, serum biochemistry, and histopathology data did not show any significant differences between control and treated groups.

Clinical biochemistry values Tables (4.1, 4.2, 4.3 and 4.4) of effects of *G. umbrosus* extract on the kidney and liver in male and female rats were within the range of the control animals tested, the values expressed as mean ± S.E.M. There are no significant differences between groups and the significant value at $p < 0.05$.

Histological sections values Figure (4.1) of the liver and kidney from the acute toxicity test. There is no significant difference in the structures between the treated and control groups (Hematoxylin and Eosin stain, 20 x magnifications).

Table 4.1: Effects of *G. umbrosus* extract on kidney biochemical parameters in male rats

Dose	Sodium (mmol/L)	Pottasium (mmol/L)	Chloride (mmol/L)	CO_2 (mmol/L)	Anion gap (mmol/L)	Urea (mmol/L)	Creatinine (µmol/L)
Vehicle (CMC)	141.33 ± 0.58	4.98 ± 0.04	103.88 ± 0.82	24.12 ± 0.54	18.75 ± 0.46	5.45 ± 0.45	34.83 ± 2.17
LD (2 g/kg)	142.05 ± 0.55	5.02 ± 0.09	105.29 ± 1.02	22.67 ± 0.81	18.55 ± 0.62	6.06 ± 0.83	33.87 ± 2.29
HD (5 g/kg)	143.14 ± 0.68	4.91 ± 0.06	104.35 ± 0.54	23.90 ± 0.64	19.15 ± 0.45	5.63 ± 0.37	35.05 ± 2.26

Table 4.2: Effects of *G. umbrosus* extract on kidney biochemical parameters in female rats

Dose	Sodium (mmol/L)	Pottasium (mmol/L)	Chloride (mmol/L)	CO_2 (mmol/L)	Anion gap (mmol/L)	Urea (mmol/L)	Creatinine (µmol/L)
Vehicle (CMC)	141.87 ± 0.42	4.83 ± 0.14	105.78 ± 0.67	23.33 ± 0.41	18.00 ± 0.25	7.95 ± 0.33	41.76 ± 2.75
LD (2 g/kg)	142.07 ± 0.56	4.55 ± 0.16	105.85 ± 0.65	22.65 ± 0.42	17.49 ± 0.46	7.97 ± 0.49	42.00 ± 2.36
HD (5 g/kg)	142.15 ± 0.47	4.63 ± 0.18	107.03 ± 0.53	21.96 ± 0.75	17.67 ± 0.48	8.31 ± 0.68	43.13 ± 2.24

Table 4.3: Effects of *G. umbrosus* extract on liver biochemical parameters in male rats

Dose	Total protein (g/L)	Albumin (g/L)	Globulin (g/L)	TB (μmol/L)	CB (μmol/L)	AP (IU/L)	ALT (IU/L)	AST(IU/L)	GGT (IU/L)
Vehicle (CMC)	64.33 ± 1.26	11.19 ± 0.17	53.17 ± 1.28	2.00± 0.00	1.00 ± 0.00	108.83 ± 4.13	43.17 ± 2.91	171.83 ± 6.38	3.67 ± 0.33
LD (2 g/kg)	63.75 ± 1.19	11.05 ± 0.45	52.33 ± 1.26	2.00 ± 0.00	1.00 ± 0.00	98.83 ± 5.25	42.96 ± 2.70	172.17 ± 6.35	3.50 ± 0.51
HD (5 g/kg)	65.02 ± 2.65	11.30 ± 0.43	53.02 ± 1.25	2.00 ± 0.00	1.00 ± 0.00	102.67 ± 5.17	44.02 ± 1.85	174.28 ± 5.26	3.22 ± 0.44

Table 4.4: Effects of *G. umbrosus* extract on liver biochemical parameters in female rats

Dose	Total protein (g/L)	Albumin (g/L)	Globulin (g/L)	TB (μmol/L)	CB (μmol/L)	AP (IU/L)	ALT (IU/L)	AST (IU/L)	GGT (IU/L)
Vehicle (CMC)	60.45 ± 1.25	9.62 ± 0.49	52.02 ± 1.40	2.17 ± 0.17	1.00 ± 0.00	153.00 ± 6.35	50.08 ± 1.62	172.95 ± 6.13	3.26 ± 0.25
LD (2 g/kg)	58.86 ± 0.86	8.81 ± 0.38	50.71 ± 1.21	2.13± 0.16	1.00 ± 0.00	154.17 ± 8.10	48.33 ± 0.58	174.23 ± 5.14	3.65 ± 0.42
HD (5 g/kg)	60.15 ± 1.05	9.17 ± 0.46	50.33 ± 1.24	2.02 ± 0.13	1.00 ± 0.00	155.00 ± 7.04	47.87 ± 1.55	175.15 ± 7.02	3.37 ± 0.18

In Tables (4.1, 4.2, 4.3 and 4.4) all values expressed as mean ± S.E.M. TB: Total bilirubin; CB: conjugated bilirubin; AP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: G-glutamyl Transferase.

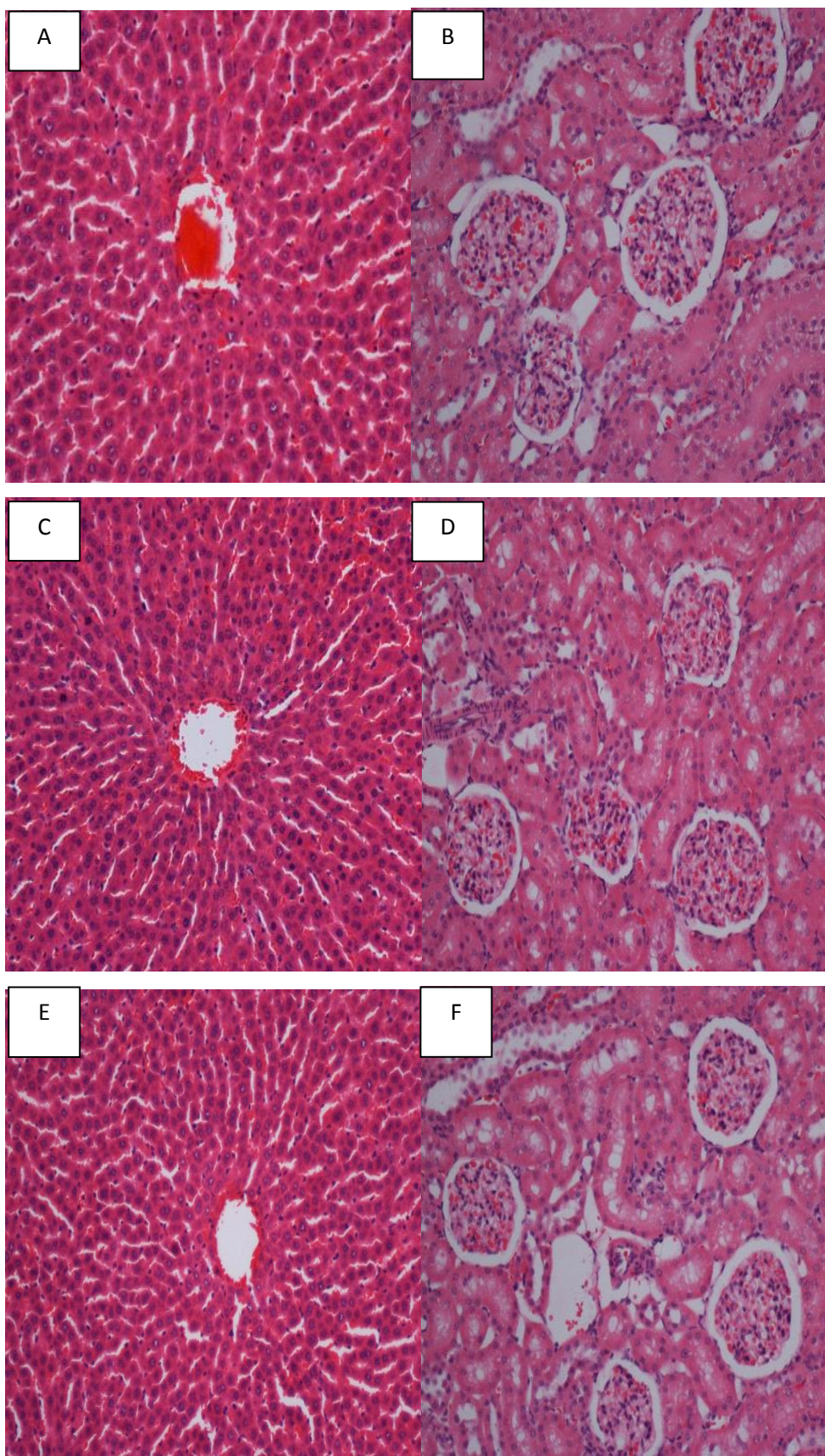## 4.2 Histological sections of the liver and kidney from the acute toxicity test

Figure 4.1: Histological sections of the liver and kidney from the acute toxicity test. (A and B) Rats treated with 5 ml/kg of the vehicle (CMC). (C and D) Rats treated with 2 g/kg (5 ml/kg) of the *G. umbrosus* extract. (E and F) Rats treated with 5g/kg (5 ml/kg) of the *G. umbrosus* extract

4.3 Wound healing activities

Grossly, wounds dressed with *G. umbrosus* treated group or with reference standard control showed considerable signs of dermal healing and significantly healed faster compared to the group received the placebo control treatment gum acacia in normal saline (Table 4.5 and Figure 4.2).

Table 4.5 showed the effects of *G. umbrosus* percentage of wound healed on day10 post-surgery. Throughout the experiment, the percentage of healing in placebo control group wound was significantly lower than those of *G. umbrosus* extract-treated group and reference standard control wounds.

Histology of wound area on day 10 post-surgery that dressed with *G. umbrosus* extract showed comparatively less scar width at wound closure compared to the placebo-treated group and the granulation tissue of wound area contained comparatively few inflammatory cells, and more collagen and proliferating blood capillaries (angiogenesis) compared with placebo-treated group (Figure 4.5).
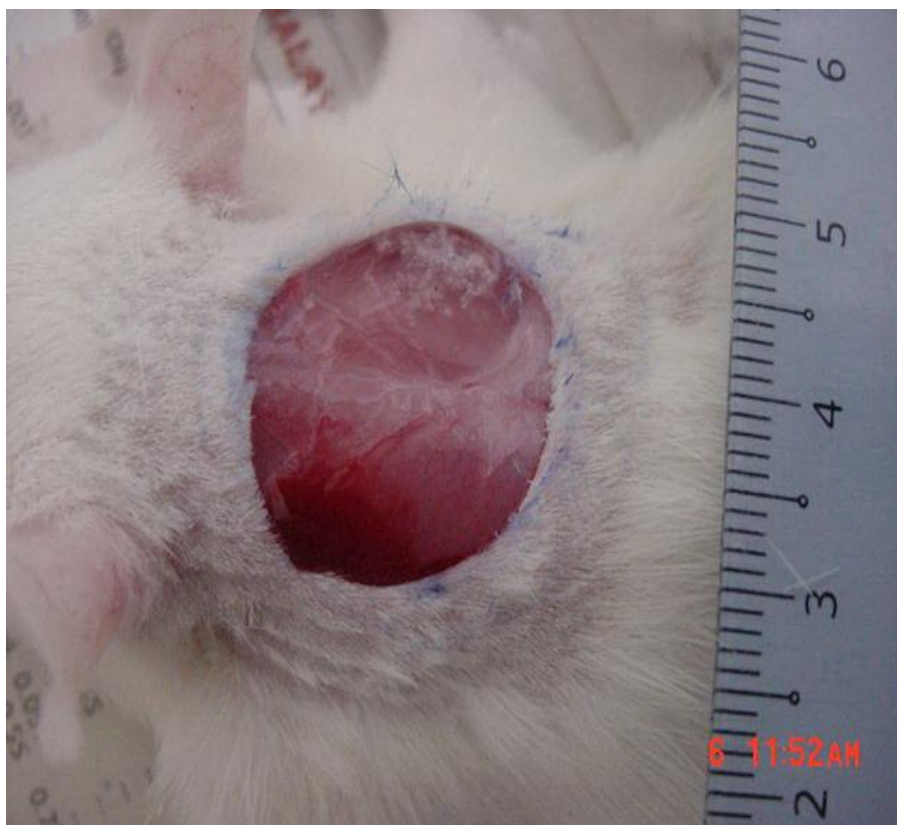

Figure 4.2: Macroscopically appearance of wound (2 cm diameter) on the first day post-surgery

4.4 Time required for wound healing by *G. umbrosus* leaf extract in rats

Table 4.5: Time required for wound healing by *G. umbrosus* leaf extract in experimental rats. All values were expressed as mean and ± standard error mean. Mean with different superscripts are significantly different (P<0.05)

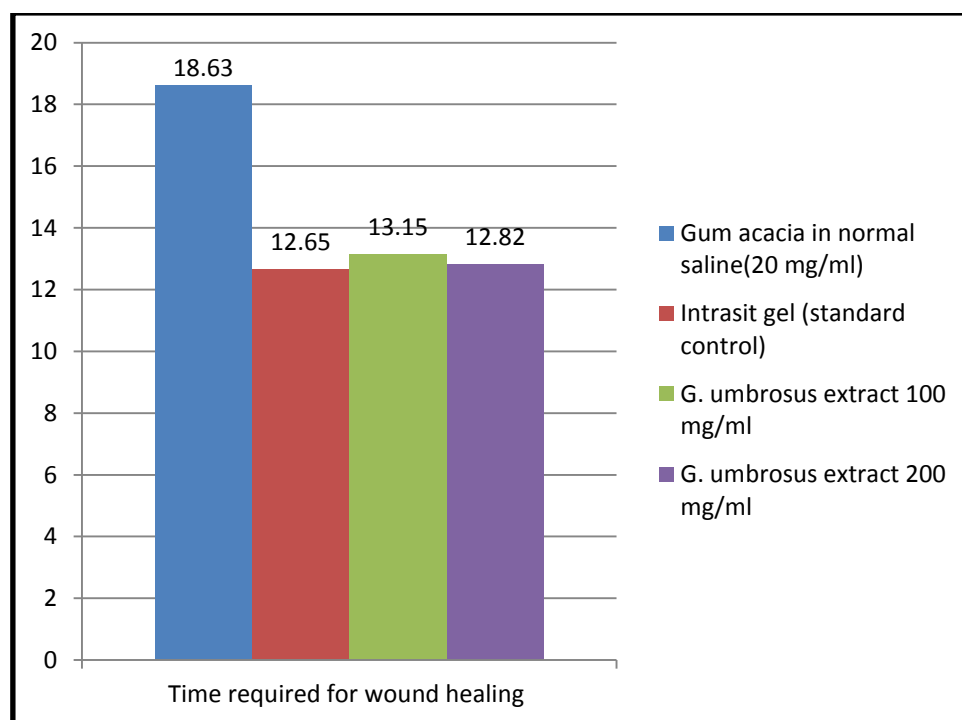| Animal group	No. of animals	Type of dressing (twice daily) (0.2 ml/animal)	Healing time (days) (Mean ± S.E.M)
1	9	Gum acacia in normal saline(20 mg/ml)	18.63 ± 0.81
2	9	Intrasit gel (standard control)	12.65 ± 0.21
3	9	*G. umbrosus* extract 100 mg/ml	13.15 ± 0.46
4	9	*G. umbrosus* extract 200 mg/ml	12.82 ± 0.14

Figure 4.3: Time required for wound healing by *G.umbrosus* leaf extract in experimental rats

4.5 Wound closure

The rate of wound healing activities was evaluated by a blind observer unaware of the experimental protocol. 200 mg/ml of *G. umbrosus*-treated group showed smallest wounds closure on day 10 post-wounding compared with the control rats (Figure 4.4). Gel treated rats has significantly ($p<0.05$) less wound area on day 10 of healing compared with control group, but the wound areas of rats treated with 100 mg/ml *G. umbrosus* were significantly smaller than those of control group (Figure 4.4 A). Throughout the experiment, the percentage healing in the control group was significantly lower than those of Intrasite gel, plant extract treated group (Figure 4.4 B). The wounds of rats treated with plant extract comparatively increased the rate of wound contraction when compared with the control group and the wound in plant-treated groups were clean and showed bright red healthy granulation tissue (Figure 4.4C). In contrast, the wounds in control rats were generally unclean surface (Figure 4.2). *G. umbrosus* treated group had reduced wound size on day 5 compared with the wounds in control group. However, the percentage healing in Intrasite gel or *G. umbrosus* was significantly more than control group (Figure 4.4D).
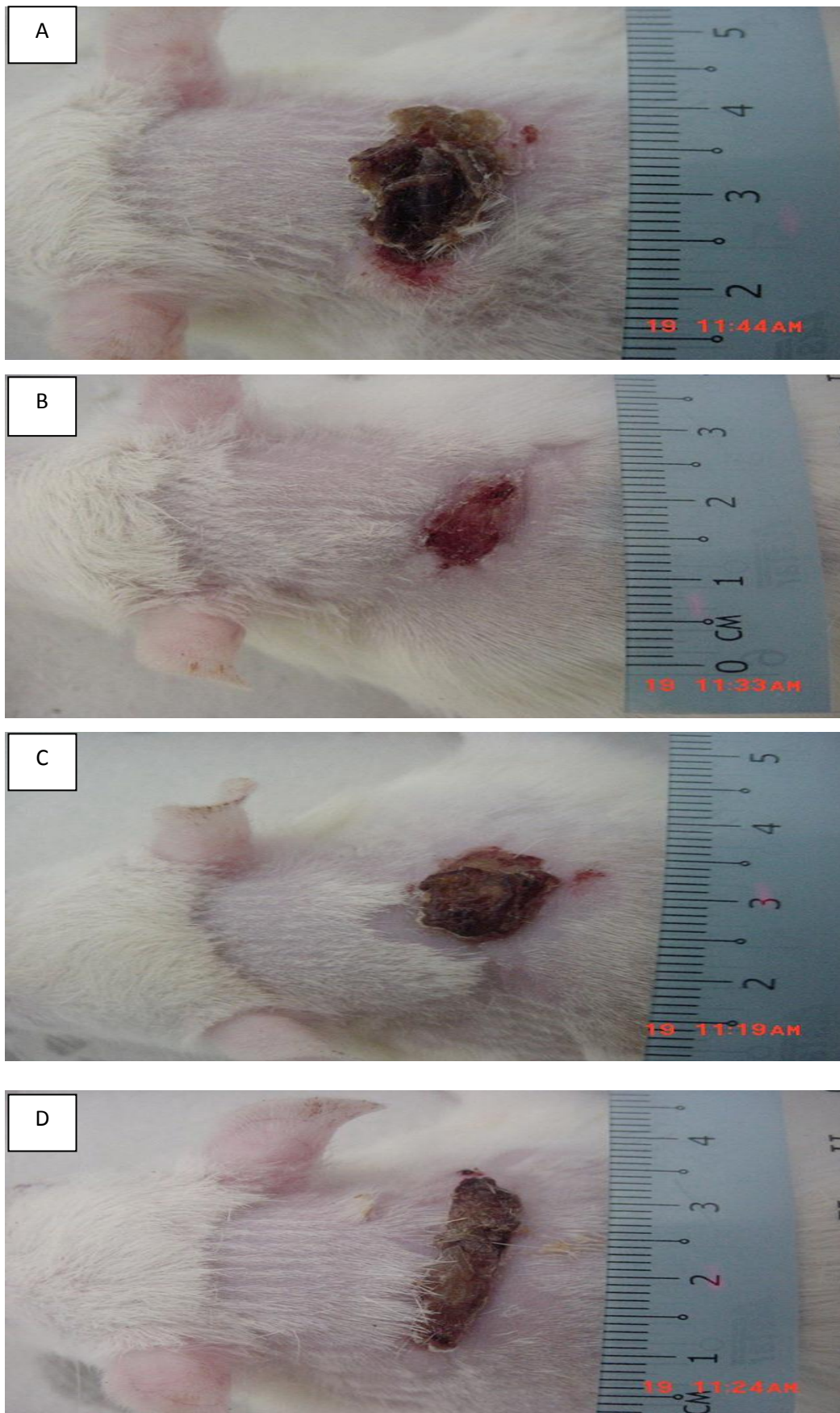
Figure 4.4: Photo macroscopically appearance of wound healing area on day 10-post-surgery in rats. (A) Topical application of 0.2 ml of vehicle, gum acacia in normal saline (20 mg/ml), showing incomplete wound healing. (B) 0.2 ml Intrasite showed complete wound healing compared with control group. (C) 0.2 ml *G. umbrosus* extract 100 mg/ml showed significant complete wound healing compared with control group. (D) 0.2 ml 200 mg/ml of *G. umbrosus* -treated group complete wound healing compared with control group

4.6 Histology of wound tissue of skin

The histology of wound of skins on day 10 post-surgery was evaluated by an observer blinded to the experimental protocol.

In 20 mg/ml G. *umbrosus*-treated groups, the wound enclosures were smaller compared with control group (gum acacia).

The granulation tissue contained comparatively few inflammatory cells, more collagen and proliferating the blood capillaries compared with control group. *G. umbrosus*-treated groups stimulated and enhanced the faster lay down of collagen fibers and new blood vessels in granulation tissue than control group (Figure 4.5).
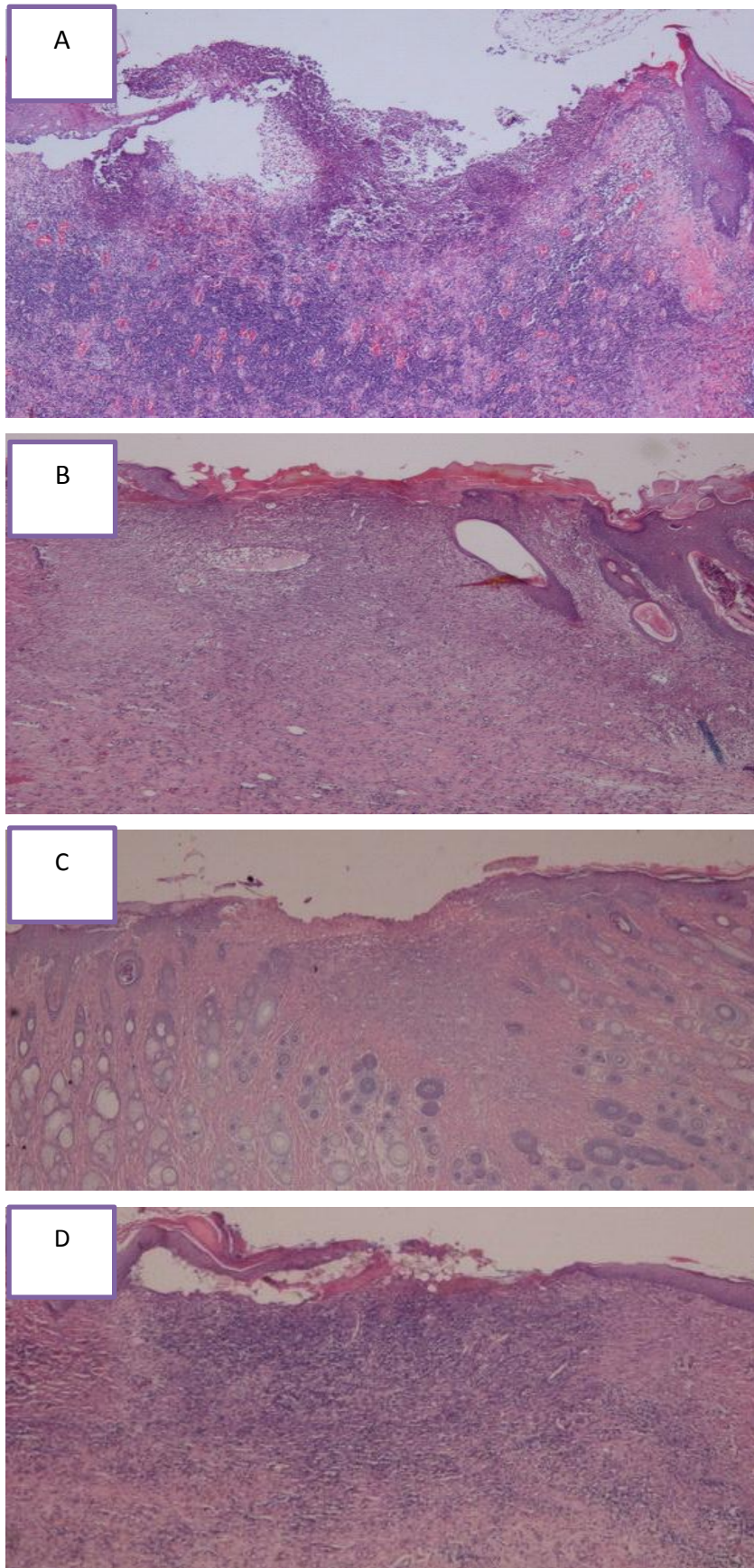
Figure 4.5: Histological section of wound enclosure on day 10-post-surgery in rats. (A)Topical application of 0.2 ml of vehicle, gum acacia in normal saline (20 mg/ml), showed wide wound closure (B) 0.2 ml of Intrasite gel showed comparatively smaller wound enclosure compared to control group (C) 0.2 ml *G. umbrosus* extract 100 mg/ml showed significant small wound closure area compared with control group. (D) 0.2 mg/ml *G. umbrosus* extract 200 mg/ml showed significant small wound closure area compared with control group (H & E stain 2x)

4.7 Antioxidant Assay in vitro

The DPPH free radical scavenging results of the positive control and plant extracts are expressed as a percentage of inhibition. Based on the values calculated from the linearity curves, the ethanol extracts showed a higher scavenging percentage than the aqueous extracts, there is a different correlation between antioxidant activity and TPC showed a positive correlation between TPC and DPPH for ethanolic extract (Figure 4.6).
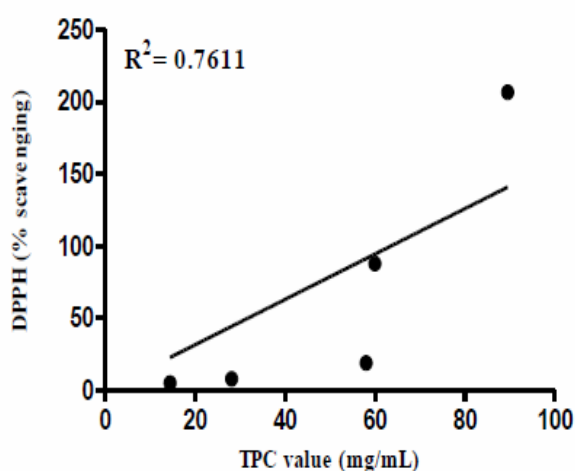

Figure 4.6: Correlation between TPC and DPPH scavenging percentage

The FRAP showed the highest reducing power for both ethanol and aqueous extract, respectively, and was not significantly different with BHA. It is also found that there is a high correlation between TPC and FRAP (Figure 4.7).
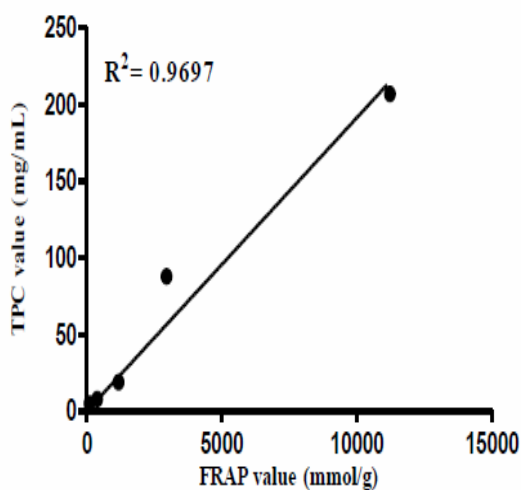

Figure 4.7: Correlation between total phenolic content and FRAP reducing power

4.7.1 Antioxidant enzymes and MDA levels in wound tissue

GPx and SOD activities and MDA levels were calculated, the number of living cells present the amount of protein in the healing wound.

Results showed that both enzymes were significantly elevated in rats receiving gels compared with controls (Figure 4.8). However, higher levels were observed with rats treated with Intrasite gel. There was significant reduction in MDA levels observed in tissue taken from rats treated with 200 mg/ml extract (Table 4.6).

Table 4.6: Antioxidant enzymes and MDA levels in wound tissue, Intrasite gel, 100 mg/ml and 200 mg/ml *G. umbrosus* extract treated rats.

Treatment	No. of animals	GPx (µ/g protein	SOD (u/mg protein	MDA (nmol/g protein)
Gum acacia	9	13.33 + 0.45	18.17 + 0.67	110.66 + 4.23
Intrasite gel	9	32.77 + 1.11	48.54 + 2.12	15.07 + 0.38
100 mg *G. umbrosus*	9	25.11 + 0.9	30.27 + 1.19	25.01 + 1.26
200 mg G. umbrosus	9	30.82 + 1.04	42.98 + 1.25	18.77 + 1.24

All values were expressed as mean and ± standard error mean. Mean in columns with different letters were significantly different (*P<0.05*).
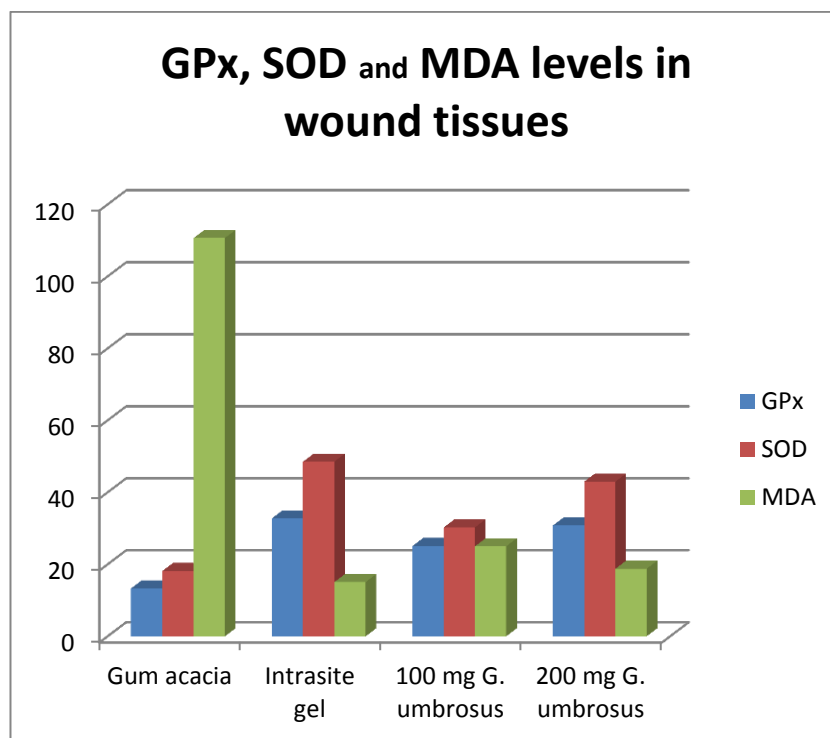
Figure 4.8:GPX,SOD and MDA levels in wound tissue, Glutathion peroxidase (GPx) and superoxide dismutase (SOD) activities and Malondialdehyde (MDA) levels in tissue homogenates of day-10 dermal wounds of control.

CHAPTER FIVE

5. DISCUSSION

It is important to note that throughout the period of wound treatment, the *G. umbrosus* extract did not cause irritation or pain to the animals as the rats neither show any sign of restlessness nor scratching/biting of wound site when the extract were applied(Muhammad and Muhammad, 2005).

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state. Wound contracture is a process that occurs throughout the healing process, commencing in the fibroblastic stage whereby the area of the wound undergoes shrinkage. The cellular proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction resulting in a smaller amount of apparent scar tissue (Wasman *et al*., 2010).

The results of the current study showed that topical application of *G. umbrosus* extract significantly accelerated the rate of wound healing, and in histology, granulation tissue contain comparatively less inflammatory, more collagen and angiogenesis. This is in line with the results of Abdulla (2009) and Mahmood (2010) who showed that wound treated with plant extract of granulation tissue contain less inflammatory, more collagen and angiogenesis.

The effects of wound healing may be due to regulation of collagen expression and increase in tensile strength of the wounds (Abdulla *et al*., 2010; Midwood *et al*., 2004). Similarly, enhanced healing activity has been attributed to collagen formation and angiogenesis (Abdulla *et al*., 2009; Mahmood *et al*., 2010).

Collagen plays a central role in the healing of wounds and it is principal component of connective tissue and provides a structural framework for the regenerating tissue (Bonte

et al., 1994). Angiogenesis in granulation tissues improves circulation to the wound site thus providing oxygen and nutrients essential for the healing process that include-re-epithelization (Suguna *et al*., 1996). Stimulate epithelial cell proliferation and angiogenesis are important for wound healing process (Diegelmann *et al*., 1986). Habibipour (2003) showed that histological analysis of the treated wound contained a large amount of fibroblast proliferation, collagen synthesis, and neovascularization, which resulted in an increased wound tensile strength and accelerated healing wound.

In case of the antioxidant parameters, rats treated with the aqueous extracts of *G. umbrosus* showed significant increase in the activity of SOD catalase and GSH with a decrease in MDA level in granulation tissue compared with controls. These enzymes are known to quench the super oxide radical and thus prevent the damage of cells caused by free radicals (Liu *et al*., 1997). Collagenation seen under the influence of this plant extract may be because of the presence of flavonoids, which is responsible for the free radical scavenging activity which is believed to be one of the most important of wound healing (Devi Priya and Shyamala Devi, 1999).

Phytochemical constituents present in the *G. umbrosus* extract may be responsible for wound-healing activity. Previous studies with plant extracts have shown that constituent like flavonoids are known to promote the wound-healing process mainly due to their antimicrobial properties, which appeared to be responsible for wound contraction and increased rate of epithelialization (Akowuah *et al*., 2002). Flavonoids are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving vascularity. Hence, any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibers formation by increasing the strength of collagen fibers, increasing the circulation, preventing the cell damage and by promoting the DNA synthesis (Buntrock *et al*., 1982). Mechanisms of wound healing may be contributed to stimulate the production of antioxidants in wound site and

provides a favorable environment for tissue healing (Habibipour *et al*., 2003). *G. umbrosus* extract has shown antioxidant activity (Yam *et al*., 2008). It has been reported that antioxidants may play a significant role in the wound healing process and may be important contributory factor in the wound healing property (Habibipour *et al*., 2003). Antioxidants have been reported to play a significant role in the wound healing process and significantly improve wound healing and protect tissues from oxidative damage (Shukla *et al*., 1999; Getie *et al*., 2002).

G. umbrosus contains a wide array of free radical scavenging molecules and flavonoids were the major naturally occurring antioxidant components in this plant (Martin, 1996; Shukla *et al*., 1999). Flavonoids can scavenge for the reactive oxygen species (super-oxide anions) and free radicals produced by ethanol. These reactive intermediates are potentially implicated in delayed wound healing (Lewis and Hanson, 1991; Akowuah *et al*., 2009). *G. umbrosus* has been shown to contain anti-inflammatory activity and it is speculated that the acceleration of wound healing potential exerted by this plant extract could be attributed to its anti-inflammatory activity (Iskander *et al.*, 2002).

To determine the safety of drugs and plant products for human use, the toxicological evaluation is carried out in various experimental animals to predict toxicity and to provide guidelines for selecting a 'safe' dose in humans Liver and kidney of the treated rats did not show significant change as compared to the control group. Clinical biochemistry values were within the range of the control animals tested and similar to some of the control reference values published elsewhere (Witthawaskul *et al*., 2003). The highest dose of *G. umbrosus* extract which did not cause any toxicity was 5 g/kg body weight suggesting that this plant is relatively non-toxic since in acute toxicity studies. The product is considered of non-toxic if no deaths are registered after 10 days of observation and no clinical signs of toxicity are observed at doses at or below 5 g/kg (Brock *et al*., 1995).

In the present study, tissue homogenate from wounds treated with *G. umbrosus* extract showed significant antioxidant activity by decreasing the levels of MDA and by elevating the levels of GPx SOD in response to oxidative stress. Free radicals and reactive oxygen species (ROS) are continuously produced in the human body. These oxygen species are caused the cells damage. Therefore, tissues must be protected from oxidative injury through intracellular as well as extracellular antioxidants (Halliwell, 1990).

Reduced activities of SOD and GPx in tissue wound homogenates in control rats have been observed in our study. Superoxide and hydroxyl radicals are important mediators of oxidative stress that play vital role in some clinical disorders. Any compound, natural or synthetic with antioxidant activities might contribute towards the total/partial alleviation of such damage. Therefore, removing superoxide and hydroxyl radical could contribute to defense of a living body against disease (Gao *et al*., 1998).

Reduced activities of SOD and GPx were observed in the tissue homogenate of diabetic control rats. This might have resulted from their utilization for the decomposition of superoxide anion generated by lipid peroxidation. Lower activities of these enzymes may result in a number of deleterious effects. GPx thought to be an important factor in cellular function and defense against oxidative stress. It was found that dietary GPx suppresses oxidative stress *in vivo* in prevention of diabetic complication (Osawa and Kato, 2005).

 Lipid peroxidation is found to be an important pathophysiological event in a variety of diseases including diabetes (Ajitha and Rajnarayana, 2001). It is well known that MDA from lipid peroxidation reacts with DNA bases and induces mutagenic lesions (Benamira *et al*., 1995).

Pratibha (2006) showed that the activated oxygen species can in turn to induce cellular events such as enzyme inactivation, DNA strands cleavage and also membrane lipid peroxidation.

CHAPTER SIX

6. CONCLUSION

These results suggest that plant extract has a beneficial effect and plays major roles in wound healing. Topical application of this plant accelerates wound healing in rats, and this could be due to increase in antioxidant (SOD and GPx) on wound tissue and decreases in the MAD level compared to controls. Our documented findings suggested the use of *G. umbrosus* for the treatment and management of wounds. Such findings encourage further investigation in order to be greater equipped to evaluate the wound healing activity of *G. umbrosus* in rats.

G. umbrosus extract showed remarkable wound healing activity grossly, and histology of wound area on day10 post-surgery showed less scar on the wound enclosure and granulation tissue contain markedly less inflammatory cells and more fibroblast and collagen fiber, and blood capillaries compared to gum acacia-treated rats.

The acute toxicity profile of this plant could be considered favorable judging from the absence of adverse clinical manifestations. Further studies with purified constituents are needed to understand the complete mechanism of wound healing activity of *G. umbrosus* extract.

Finally concluded that *G. umbrosus* leaf extract orally administered to rats was safe and that is no drug-related toxicity was detected.

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APPENDIX

A. Histology technique:

- Specimen preparation.
- Automated tissue processing.
- Tissue blocking.
- Sectioning.
- Staining.
- Mounting.

B. Specimen preparation:

- Label all the biopsies properly.
- The tissue biopsies were fixed in 10 % buffered formalin solution as possible to prevent autolysis.
- Trimming
- Trim the interest tissue area appropriate.
- Size 1 cm x 1 cm, 5 mm thickness.
- Put in the cassette, closed.
- Labeled properly.
- Put into the fixative again (10% buffered formalin).
- Place the train tissue in the cassette and labeled properly.
- Then, put the cassette containing the tissue in the fresh 10 % buffered formalin once against for overnight.

C. Steps of tissue processing with automated tissue processing machine

10% Buffered formalin (1 hour)
70% Ethanol (1 hour)
95 % Ethanol I
95 %Ethanol II
95 % Ethanol III
Absolute alcohol I (1 hour)
Absolute alcohol II (1 hour and 30 minutes)
Abs.ethanol + Xylene (1 hour)
Xylene   I (1 hour and 30 minutes)
Xylene II (1 hour and 30 minutes)
Paraffin wax I (1 hour)
Paraffin wax II (1 hour)

D. Tissue blocking

- The tissue were aligned and oriented in the mold properly to facilitate the sectioning process.
- The paraffin wax was poured into the mold until cover the whole tissues.
- Then the mold was placed on cold plate area to harden the wax.

E. Sectioning

- The tissue blocks were sectioned by using the rotary microtome at the thickness of 5 µm thick.
- Then the ribbon sectioned tissues were floated on the warm water bath.
- The sections were picked up one by one by slides.

F. Haematoxylin and eosine (H& E) staining

i. Reagent:

- Harris Hematoxylin working solution.
- Eosin working solution.
- 0.5% acid alcohol.
- 2% sodium acetate.
- 80% alcohol.
- 95% alcohol.
- Absolute alcohol.
- Xylene.
- DPX.
- Cover slips.

ii. Method:

- Bring section to water.
- Stain in harris hematoxylin for 10 minutes.
- Wash in running water until excess blue colour removed.
- Differentiation with 0.5% acid alcohol, wash well in water and observed.
- Wash well in water.
- Dip 2 times in 2% sidium acetate.
- Wash again in running tap water for 2-3 minutes.
- Rinse the slide in 80% alcohol.
- Stain in eosin solution for 5 minutes.
- Dehydration :

95% alcohol I	5 second
95% alcohol II	2 minutes
Absolute alcohol I	2 minutes
Absolute alcohol II	2 minutes

- Put the slide in xylene for 3 times, 2 minutes each.
- Mount in DPX.
- Wipe slide to remove excess xylene.

G. Exposure to water (rehydration)

i . Materials:

- Xylene
- Absolute alcohol
- 95% alcohol
- 70% alcohol

ii. Method:

- Place slide in xylene with 2 changes, 3 minutes each.
- Drain excess xylene.
- Transfer slide to absolute alcohol for 2 minutes.
- Transfer slide to 95% alcohol for 2 changes, 2 minutes each.
- Transfer slide 70 % alcohol for 2 minutes.
- Leaves the slide in slow running water.