

1. INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterised by hyperglycemia (high blood glucose) with disturbance of carbohydrate, fat and protein metabolisms, results from defects in insulin secretion, action or both (Watkins, 2003). It affected more than 180 million individuals worldwide, leading to debilitating consequences such as vasculopathy, retinopathy and neuropathy (Watkins, 2003). More than 80 per cent of diabetes mellitus is Type-2 (non-insulin dependent) characterized by peripheral resistance to the action of insulin and decreased peripheral glucose uptake, or increased hepatic glucose output (Watkins, 2003). Type-1 (insulin-dependent) represents 5 to 10 per cent of diabetes mellitus patients and is caused by the failure to secrete insulin due to autoimmune destruction of beta-Langerhans cell. Furthermore, according to the statistics provided by the National Diabetic Information Clearinghouse where 15% of diabetic individuals suffered from diabetic foot ulcer. The impaired healing of wound led to 84% of lower leg amputation was related to DM (cited in Brem & Tomic-Canic, 2007). The hyperglycemic state in diabetic patients, especially those with peripheral vasculopathy interrupts appropriate wound healing. Poor blood circulation in the affected area leads to microbial infection and gangrene formation (Wild *et al.*, 2004). This often leads to increased morbidity and mortality. Diabetes and trauma leads to increased oxidative stress and formation of reactive oxygen species or free radicals that have been implicated in the pathophysiology of wound (Merisogulari & Bakan, 2004).

There are many treatments for wounds and effective wound management requires an understanding of the process of tissue repair and knowledge of the properties of many sophisticated dressings that are now available. Currently, the wounds in diabetic patients

are managed with topical antimicrobial creams. Besides antibiotics and debridement for non-limb threatening infection, amputation of limb is one of the worst considerations for limb-threatening infection (Frykberg *et al.*, 2006). Consequently, foot disorders are leading causes of hospitalisation for persons with DM and account for billion-dollar expenditures annually for the country. Therefore, search for the cost effective and holistic wound treatment cream for diabetic patients is emphasized.

Natural sources like plant and mushroom extracts have enormous potential as a source of both dietary protein and health-enhancing dietary supplements (Chang & Buswell, 2008). Other than health enhancing effects, phytochemicals and bioactive compounds in these natural sources could have effects in wound healing. Extracts of *Aloe vera* (Chithra *et al.*, 1998), *Catharanthus roses* flower (Nayak *et al.*, 2006) and *Ocimum sanctum* Linn (Shetty *et al.*, 2007) were found to have high antioxidant activities and enhanced wound healing in *Sprague Dawley* rats. Wound healing properties were also found in fungi, such as sacchachitin membrane made from the residue after hot water extraction of *Ganoderma tsugae* was found to accelerate wound healing similar to Beschitin (Su *et al.*, 2004). Beta-glucan from a medicinal mushroom, *Sparasis crispa* improved wound healing in streptozotocin-induced diabetic rats by directly increasing the synthesis of type I collagen.

Among the medicinal mushrooms, *Ganoderma* species are much sought out for their wide array of medicinal properties. *Ganoderma lucidum* (M.A. Curtis:Fr.) P. Karst is a medicinal mushroom, which belongs to the Polyporaceae of Aphyllophorales. Its fruiting body is called Ling zhi in China and Reishi in Japan. For hundreds of years, this mushroom has been regarded as a traditional Chinese medicine or a folk medicine used for the prevention and treatment of various human diseases, such as chronic bronchitis, hepatitis, hypertension, hypercholesterolemia, tumorigenic diseases and immunological disorders in China and other Asian countries (Lin, 2001). Again, chemical analysis of *G. lucidum* has

indicated the presence of beneficial components including polysaccharides (Zhang *et al.*, 2003); triterpenoids (Lin & Shiao, 1989) and steroids (Gan *et al.*, 1998b). It has been shown that *G. lucidum* polysaccharides have antitumor (Maruyama *et al.*, 1989; Miyazaki & Nishijima, 1981; Zhang & Lin, 1999) and immunomodulatory (Xia & Lin, 1989; Lei & Lin, 1992). Other than polysaccharides and triterpenes, total phenols were the major naturally occurring antioxidant components found in methanolic extracts of *G. lucidum* and *G. tsugae* (Mau *et al.*, 2002). However, to our knowledge the aqueous extracts of *G. lucidum* has not been investigated for its role in wound healing.

Under several situations, when the stress level exceeds defence capacity, it may induced oxidative damage, whereas the low level stress may stimulate defence network and induce adaptive response (Niki & Yoshida, 2005). There are multiple sources of oxidative stress in diabetes including non-enzymatic, enzymatic and mitochondrial pathways. Non-enzymatic sources of oxidative stress originate from the oxidative biochemistry of glucose, thus hyperglycemia can directly cause increased reactive oxygen species (ROS) generation (Halliwell & Gutteridge, 2000). Antioxidant defence system of the body consists of endogenous and exogenous antioxidants that work together at the molecular level to protect cell membranes, lipoproteins, and DNA from the damaging effects of free oxygen radicals (Halliwell *et al.*, 1989). Therefore the antioxidant capacity and oxidative indices in experimental rats can be related to wound healing in normal and diabetic rats. *In vivo* antioxidant capacity and oxidative damages during wound healing was evaluated in the serum of rats on day 16 post operation.

As plasma proteins are the critical targets for oxidants, advanced oxidation protein products (AOPP) is a novel marker of oxidative stress, detected in plasma or purified human serum albumin (HSA). Measurement of AOPP is a reliable marker to estimate the degree of oxidant-mediated protein damage and to predict the potential efficacy of

therapeutic strategies aimed at reducing such an oxidative stress (Witka-Sarsat *et al.*, 1996). Protein oxidation products and carbonyl derivatives of proteins may result from oxidative modifications of amino side chains, reactive oxygen-mediated peptide cleavage, and reactions with lipid and carbohydrate oxidation products. Studies have shown that the presence of carbonyl groups in proteins may indicate that the proteins have been subjected to oxidative (Ergul *et al.*, 2004).

Furthermore, lipids are susceptible to oxidation and lipid hydroperoxides (LHP) is the product of lipid oxidation, which may serve as the oxidation biomarker. It has been demonstrated that reactive oxygen species, free radicals and oxidative products, such as lipid hydroperoxides, participate in tissue injuries and in the progression of degenerative diseases in humans (Wijeratne & Cuppett, 2006). Lipid peroxidation is an example of oxidative damage in cell membranes, lipoproteins, and other lipid-containing structures. Peroxidative modification of unsaturated phospholipids, glycolipids, and cholesterol can occur in reactions triggered by free radical species and non-radical species. Lipid hydroperoxides are prominent non-radical intermediates whereas advanced oxidation protein products (AOPPs) are of radical-mediated advanced protein products, whose identification can often provide valuable mechanistic information (Wijeratne & Cuppett, 2006). A growing number of studies have shown a role for reactive lipid oxidation products, such as lipid hydroperoxides and its breakdown product hydroxynonenal, in the initiation of redox-sensitive signal transduction pathways (Herrlich & Bohmer, 2000). Many studies have shown that lipid peroxides and reactive oxygen species are involved in the development of a variety of diseases, including cancer, diabetes mellitus and aging. Factors that may contribute to the failure of some wounds to heal include elevated levels of oxygen free radicals and resulting products of oxidation which chemically alter the essential components in cell (James *et al.*, 2003; Moseley *et al.*, 2004). Oxidative stress in diabetic wound healing rats

was measured by the resulting oxidative products such as LHP and AOPP, related to cytotoxicity and delayed wound healing, both of which are stable markers of oxidative stress.

Wounds in particular wounds of diabetic patients are an enormous problem worldwide and becoming an overwhelming burden in the cost of healthcare system. Mushroom extract could be an alternative natural product for wound treatment. Since this medicinal mushroom can be abundantly cultivated in the green house, thus it might be a reasonable cost effective candidate for consideration. Wound healing in hyperglycaemic state is difficult and challenging.

This study was carried out with the following objectives:

- 1.1 To extract and characterise aqueous extracts of *G. lucidum*.
- 1.2 To investigate the effect of aqueous extract of *G. lucidum* on wound healing in normal and streptozotocin-induced diabetic rats.
- 1.3 To evaluate the oxidative stress of streptozotocin-induced diabetic rats during wound healing process.

2. LITERATURE REVIEW

2.1 Diabetes Mellitus

Diabetes may occur either when there's a lack of insulin or when there's a presence of the factors that could affect the action of insulin. Insufficient actions of insulin results in increasing blood glucose concentration, known as (hyperglycaemia). When there is a severe lack of insulin, many metabolic abnormalities will occur, and notably an increase in ketone bodies in the blood. Type-1 and Type-2 diabetes are the most common type of primary diabetes mellitus. It is important both clinically in assessing the different in the two groups and understanding the causes of diabetes and their treatments (Table 2.1) (Watkins, 2003).

Table 2.1: Comparison of Type-1 and Type-2 diabetes

Type-1 diabetes	Type-2 diabetes
Inflammatory of the islets (insulinitis)	No inflammatory of the islets
Islet B-cells destroyed	B-cells function
Islet cell antibodies	No islet cell antibodies
HLA related	Not HLA related
Not inherited	Inherited

Source: ABC of diabetes, 5th edition BMJ Books

(i) Type-1 diabetes

Type-1 diabetes is due to destruction of B-cells in the pancreatic islets of Langerhans with resulting loss of insulin production or absolute insulin deficiency. Type-1 diabetes can be immune mediated or idiopathic. Type-1 diabetes occurs in genetically susceptible individuals with combinations of genetic and environmental factors that will further trigger an autoimmune attack on the patients B-cells. Thus, only about one-third of the pairs are concordant for diabetes among monozygotic identical twins (Watkins, 2003).

Human leukocyte antigen (HLA) is the major histocompatibility complex antigens that are adjuncts to several types of immunological activity. Ninety per cent of Type-1 diabetic patients show either HLA-DR3 or HLA-DR4, or both together, which associated with autoimmune disease, while HLA-DR2 is protective against diabetes. In most Type-1 diabetic patients islet cell antibodies are present at diagnosis and gradually decline and disappear during the following years. Recently, more antibodies to specific proteins have been identified: and these include the antibodies to glutamic acid decarboxylase (GAD, a 64-kDa antigen); and tyrosine phosphatase (37 kDa, IA-2). The presence of three or more islet cell antibodies, such as anti-GAD antibodies, anti-IA-2 antibodies, anti-insulin autoantibodies, in a non-diabetic individual indicates an 88% chance of developing diabetes within 10 years (Wilcox *et al.*, 2009). The presence of insulinitis at the onset of Type-1 diabetes represents the role of inflammatory cells (for example, cytotoxic T-cells and macrophages) in B-cell destruction. Macrophages also produce cytokines leading to activation of lymphocytes known to be present at the onset of Type-1 diabetes. Attempts have been made to prevent the onset of Type-1 diabetes. Islet function can be preserved by immune suppression to some extent, but permanent remissions of this disease are not normally achieve, besides the treatment is too dangerous for routine use in any case (Wilcox *et al.*, 2009).

(ii) Type-2 diabetes

Type-2 diabetes (previously non-insulin dependent diabetes) ranges from those with predominant insulin resistance, to those with insulin secretory defect with relative insulin deficiency. Islet destruction is known to start several years before the clinical onset of diabetes and probably begins very early in life.

There are many causes of Type-2 diabetes, and already known to include a wide range of disorders with differing progression and point of view. The implicit mechanism is due to either the diminished insulin secretion, or an islet defect, which is associated with increased peripheral resistance to the action of insulin resulting in increased hepatic glucose output, or decreased peripheral glucose uptake. Almost certainly as many as 98% of Type-2 diabetic patients are “idiopathic”, thus, no clear causative defect has been established. Whether increasing insulin resistance or decreasing insulin secretion that occurs first is still in doubt, but in different individuals the sequence of events may vary. The most common cause of insulin resistance is obesity (Watkins, 2003).

Some adults (especially those not overweight) over 25 years of age who appear to present with Type-2 diabetes may have latent autoimmune diabetes of adulthood (LADA) and become insulin dependent. In this group of patients autoantibodies are often present. Type-2 diabetes is a slowly progressive disease: insulin secretion declines over several decades, resulting in an insidious deterioration of glycaemic control which becomes increasingly difficult to achieve. Relative insulin resistance occurs in obese subjects, possibly because of down regulation of insulin receptors due to hyperinsulinaemia. For obese subjects there is considerably increased risk of developing Type-2 diabetes (Watkins, 2003).

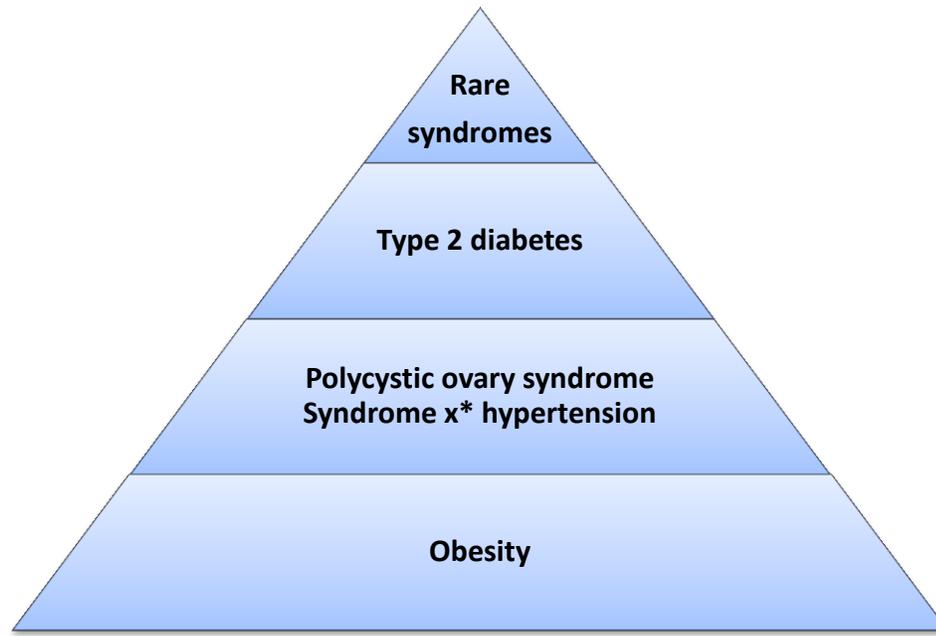


Figure 2.1: Causes of diabetes

* Syndrome x includes hyperlipidaemia, hyperinsulinaemia, and glucose intolerance

Source: (Watkins, 2003).

(iii) Gestational diabetes

Gestational diabetes mellitus (GDM) is a form of diabetes consisting of high blood glucose levels during pregnancy. It develops one in 25 pregnancies worldwide and is associated with complications to both mother and the baby. Gestational diabetes mellitus sometimes disappears after pregnancy but women with GDM and their children are at an increased risk of developing Type-2 diabetes in future. Approximately half of women with a history of GDM will develop Type-2 diabetes within five to ten years after delivery (Diabetes Care, 2004). In this group of patient, the presence of fasting hyperglycemia (>105 mg/dl or >5.8 mmol/l) may be associated with an increase in the risk of intrauterine fetal death during the last 4–8 weeks of gestation. Although uncomplicated GDM has not been associated with increased risk of fetal macrosomia, perinatal mortality, jaundice, polycythemia, hypocalcemia, and the neonatal hypoglycaemia, may occur and complicate

GDM. It is also associated with an increased frequency of maternal hypertensive disorders and cesarean delivery may be needed due to fetal growth disorders (Metzger & Coustan, 1998).

2.1.1 Prevalence of Diabetes

Diabetes mellitus is one of the most common diseases globally, it is the fourth or fifth leading cause of death in most high-income countries, and it is epidemic in many economically developing and newly industrialised countries. Diabetes is undoubtedly one of the most challenging health problems in the 21st century. Population-based diabetes studies consistently show that a substantial proportion of those found to have diabetes had not been previously diagnosed. Many people remain undiagnosed largely because there are few symptoms during the early years of Type-2 diabetes or symptoms may not be recognised as being related to diabetes. Besides diabetes, in a state of impaired glucose tolerance (IGT), where the blood glucose level is higher than normal level but not as high as in diabetes patient, is also a serious public health problem. People with IGT have a higher risk to suffering diabetes as well as an increased risk of cardiovascular disease in the near future.

The report provides estimation of the global prevalence of diabetes worldwide in the year 2011 was 366 million people and projections for 2030 will have risen to 552 million (Whitting *et al.*, 2011). 80% of people with diabetes live in low- and middle-income countries and their age are between 40 to 59 year old. Fifty per cent (183 million) people with diabetes are undiagnosed. Diabetes caused 4.6 million deaths and at least USD 465 billion dollars in healthcare expenditures in 2011 (Whitting *et al.*, 2011).

According to the report of International Diabetes Federation, the prevalence of diabetes in Malaysia was illustrated in Table 2.2.

Table 2.2: Diabetes in Malaysia in year 2012

Total adult population (1000s) (20-79 years)	17,796.66	Number of deaths in adults due to diabetes	25,859.00
Prevalence of diabetes in adults (20-79 years) (%)	11.70	Mean healthcare expenditures due to diabetes per person with diabetes (USD)	513.01
Total cases of adults (20-79 years) with diabetes (1000s)	2,082.48	Number of cases of diabetes in adults that are undiagnosed (1000s)	2,100.00

Source: International Diabetes Federation 2012, www.idf.org/diabetesatlas dated December 2013.

The same surveys showed that the prevalence of obesity also increased for adult Malaysians aged 18 years and above. Malaysia has the most number of overweight and obese people in Asia. High sugar intake among Malaysians is one of the contributing factors to the high incidence of diabetes (source: www.consumer.org.my, dated December 2013).

2.1.2 Diabetes Complications

People with diabetes have an increased risk of developing a number of serious diseases such as heart disease, high blood pressure, eyes, kidneys, nerves and teeth problems. People with diabetes also have a higher risk of developing infections in their wounds and lead to lower limb amputation (Fig. 2.2).

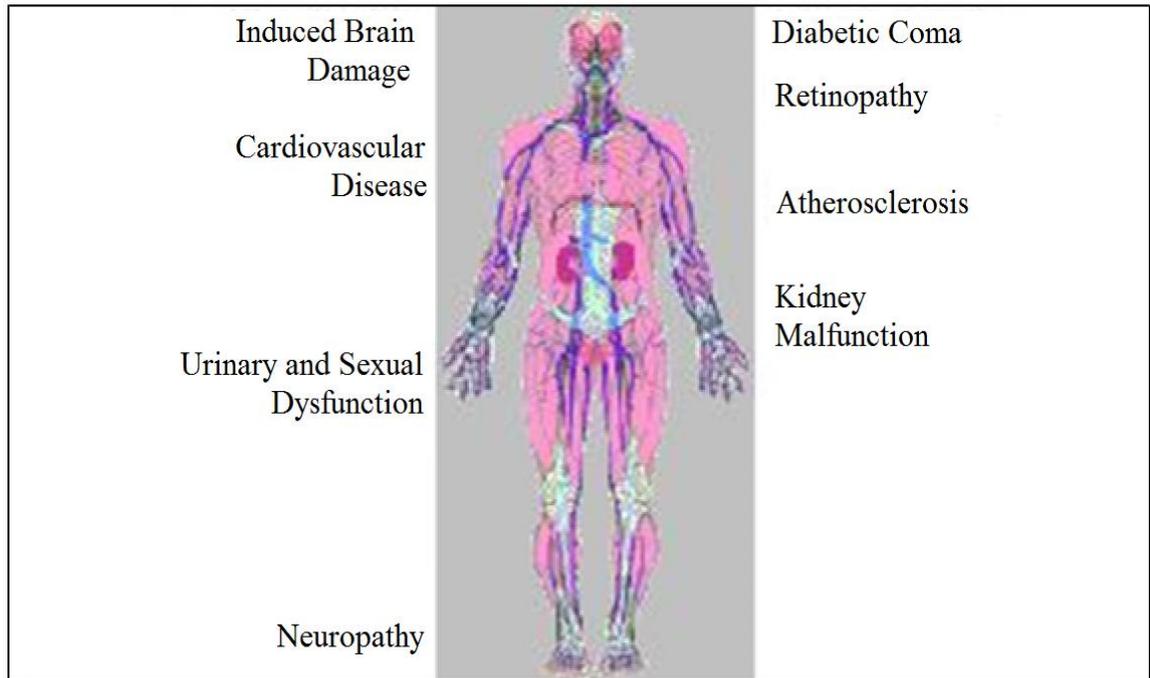


Figure 2.2: Complication of diabetes (www.montana.edu/wwwai/imsd/diabetes/comp.htm, dated 1st September 2013).

(i) Cardiovascular disease

Cardiovascular diseases (CVD), comprising coronary heart diseases (CHD) and cerebro-vascular diseases, are the common cause of death, accounting for 21.9 per cent deaths, and will increase to 26.3 per cent by 2030 (WHO press, 2008). Type-2 diabetes mellitus (T2DM) has a distinctive association with CHD with two to four-fold higher risk of developing coronary disease than people without diabetes, and CVD accounts for 65 to 75 per cent of deaths in people with diabetes (Moss *et al.*, 1991). In addition, a complex processes such as oxidative stress, atherogenicity of cholesterol particles, augmented haemostatic activation, abnormal vascular reactivity and renal dysfunction have been proposed as features characteristic of T2DM that may confer excess risk of CHD (Deedwania & Fonseca, 2005).

(ii) Diabetic nephropathy

Kidney disease is caused by the damage in the small blood vessels that act as a filter for blood. Kidney disease is more common in people with diabetes than in those without diabetes. Maintaining normal levels of blood glucose and blood pressure can lower the risk of kidney disease. Once kidneys fail, the person must choose whether to do dialysis or to get a kidney transplant. High levels of blood glucose make the kidneys filter overloaded and finally fail. Leakage of protein in the urine, microalbuminuria or macroalbuminuria can be detected in patient with diabetic nephropathy (Watkins, 2003).

(iii) Diabetic neuropathy

Diabetes can cause damage to the nerves throughout the body when blood glucose and blood pressure are too high. Among the most commonly affected area is the feet. Nerve damage in this area is called peripheral neuropathy and this can lead to pain, tingling and loss of feeling. Injuries can go unnoticed, thus cause serious infections which lead to amputations. Diabetic people have a risk of amputation 25 times greater than that of people without diabetes. Diabetic patients who develop microalbuminuria or proteinuria, has the greatest risk of large vessel disease, which is associated with widespread vascular damage. Arterial narrowing tends to be in more distal in diabetic people than in non-diabetic people. There are also substantially increased of medial arterial calcification (Monckeberg's sclerosis) in patients with renal impairment and in those with neuropathy. The potential of pharmacological agents to alter the course of neuropathy has been extensively studied, but so far none of the drugs investigated has demonstrated convincing clinically significant benefit (Watkins, 2003).

Reduced sensation in the feet may result in unnoticed injury from ill-fitting shoes, nails or stones, or burns from hot water bottles or fire. Self-inflicted wounds from crude

attempts at chiropody are dangerous because they could be infected. Microorganism such as streptococci, staphylococci, anaerobic bacteria, and gram negative organisms can secondarily infected ulcers by which can lead to osteomyelitis, cellulitis, and abscess formation. Gangrene of the toe may just starts with a sepsis complicating apical toe ulcers that can spread into in situ thrombosis of the digital arteries. The foot is always warm, with bounding pulses (Plate 2.1) (Watkins, 2003).



Plate 2.1: Neuropathic Ulcer and Ischaemic Ulcer
Source: ABC of Diabetes, 5th Edition BMJ Books (Watkins, 2003).

(iv) Diabetic retinopathy

Most people with diabetes will develop some form of eye disease (retinopathy) causing reduced or total loss of vision. Consistently high levels of blood glucose, high cholesterol, high blood pressure can cause retinopathy complication. It can be prevented through regular eye check-up and keeping glucose and lipid levels at or close to normal. Diabetic retinopathy is a systemic disease which affects up to 80 per cent of all patients who have had diabetes for 10 years or more (Watkins, 2003).

(v) Pregnancy complications

Women with any type of diabetes during pregnancy risk a number of complications if they do not carefully monitor and manage their condition. Congenital malformations and perinatal mortality is increased for both types of diabetes. The most common complication is macrosomia a result of poor glycemic regulation. High blood glucose level can lead to the foetus putting on excess weight. This can cause problems in delivery, trauma to the child and mother, and sudden drop in blood glucose for the child after birth. Children exposed to high blood glucose in the womb for a long period are at higher risk of developing diabetes in the future. Fetal malformations and macrosomia as well as other related complications can be avoided by maintaining glycemia within the normal values (Novak, 2004).

2.1.3 The Influence of Diabetes on Wound Healing

Diabetes is a systemic disorder that affects almost all body systems, either directly or indirectly through its complications. Among the acute complications, acute metabolic derangements, urinary tract infections, skin wound healing and other infections and side effects of drugs are important. The major chronic complications are retinopathy, nephropathy, neuropathy, ischaemic cerebrovascular disease, heart disease, peripheral arterial disease and skin lesions. Among these, peripheral arterial disease is one of the major morbidities. In the US, 35-45% of all limb amputations are performed on people with diabetes. Type-2 diabetes has a stronger association with these morbidities than Type-1 diabetes does (Watkins, 2003).

The hyperglycaemia (high blood glucose) associated with diabetes can cause tissue damage in two ways. The first pathway is the intracellular hyperglycaemia caused by increased flux through different metabolic pathways, which can adversely affect cellular

functions. This is the underlying mechanism of early diabetic cataracts and peripheral neuropathy. The second and more crucial pathway for long-term complications in diabetes is the non-enzymatic glycation of proteins. Glucose chemically attaches to the amino group of proteins without the involvement of enzymes to form stable protein products known as 'Amadori products' which accumulate over the surface of structural proteins, circulating proteins and cell membranes (Khan, 2005).

Proteins with a longer half-life, such as fibrin, collagen, haemoglobin, and albumin accumulate advanced glycation end products, which formed slowly from Amadori products through series of further reactions. The extent of these reactions depends on the concentration of glucose, the duration of hyperglycaemia and the half-life of these proteins. This non-enzymatic glycation can affect a number of physiological processes in the body, ranging from enzymatic activity and binding of regulatory molecules to cross-linking of proteins and susceptibility to proteolysis (Khan, 2005).

Non-enzymatically glycosylated collagen binds soluble proteins to form in situ immune complexes characteristic of diabetic nephropathy. Similarly, thickening of basement membrane in the microcirculation can lead to ischaemia and decreased tissue perfusion, which results in delayed wound healing. The important proteins from a wound healing perspective that are affected by non-enzymatic glycation are collagen, fibrin and keratin (Khan, 2005).

Non-enzymatic glycation of fibronectin decreases its ability to bind to collagen, gelatin and heparin. This protein postulated, however, defects in wound healing are caused by the hyperglycosylation of the locally synthesised cellular fibronectin, not due to the effect on plasma fibronectin. Fibronectin is the major glycoprotein secreted by fibroblasts during initial synthesis of extracellular matrix (ECM) proteins. It promotes re-epithelialisation and acts as a transduction agent in wound contraction (Diegelmann &

Evans, 2004). One of the first reports to analyse the molecular status of ECM molecules in chronic wounds assessed the stability of fibronectin and vitronectin in fluids collected from chronic wounds (Wysocki *et al.*, 1990).

The components of the ECM provide strength, compressibility and elasticity in normal skin. In severe wounds the provisional wound matrix, containing fibrin and fibronectin, plays several crucial roles, including contributing a scaffold to direct cells into the injury as well as stimulating them to proliferate, differentiate and synthesize new ECM. As healing takes place, the starting ECM of the scar undergoes remodelling and eventually the injured tissue is repaired rather than regenerated because the architecture of the scar never completely reproduces the pre-wound architecture of the skin tissue. In some wound healing fails to progress through the sequential phases and a chronic wound develops. These wounds are often characterized by increased levels of inflammatory cells that are associated with elevated levels of proteases; these appear to degrade the ECM components, receptors, and growth factors that are necessary for healing (Schultz *et al.*, 2005). The ECM is the largest component of normal skin and gives the skin its unique properties of elasticity, tensile strength and compressibility. Understanding the importance of re-establishing a functional ECM in chronic wounds has led to technical advances and the development of products that reduce excessive protease levels or contribute functional ECM proteins, thus promoting the healing process (Schultz *et al.*, 2005).

Insulin, an anabolic hormone which promotes protein synthesis and utilisation of glucose, while diabetes affects the metabolism of carbohydrates, fats, and proteins which play an important role in cellular activities, proliferation, and migration and wound healing (Cooper, 1990). When insulin availability is disrupted; all chronic wounds which begin as acute wounds with a fibrin clot, but rather than progressing through the four phases of healing they become 'stuck' in a lengthened inflammatory phase. It has been suggested that

this prolonged inflammatory phase causes increased levels of proteases such as matrix metalloproteinases (MMPs), elastase, plasmin and thrombin, which destroy components of the ECM and damage the growth factors and their receptors that are essential for healing (Schultz *et al.*, 2005).

2.2 Wound Healing

Wound healing, or wound repair, is the body's natural mechanism of regenerating dermal and epidermal tissue when an individual is wounded. Wound healing occurs as a cellular response to injury and involves activation of endothelial cells, fibroblasts, keratinocytes, platelets, and macrophages, (Brem & Tomic-Canic, 2007). It is a complex process involving inflammation, collagen deposition, fibroplasia, neovascularization, epithelialization, and wound contraction. Healing of wounds occurs by two physiological processes called regeneration and repair (Campbell, 2009).

In regeneration the wound heals as the lost tissue is replaced by cells from adjacent healthy tissue. To replace the cells lost as a result of the injury, mitosis occurs in these neighboring cells. This mechanism means that the tissue is restored, more or less as it was, by the process of cellular and tissue regeneration. Good cosmetic and functional results are received from this ideal form of healing (Campbell, 2009).

Repair is an efficient method of closing and 'patching' damaged tissues. The damaged specialized tissue is replaced with collagen. Collagen is the main component of fibrous scar tissue; it is a tough protein with high tensile strength. Thus, the functional and cosmetic results are poor because the original tissue is replaced with fibrous tissue. Most wounds healing process is by a combination of regeneration and repair (Campbell, 2009).

During the healing process, various growth factors and cytokines are released by these cell types that are needed to coordinate and to accelerate wound healing (Singer &

Clark, 1999). Furthermore the phases of wound healing can be divided into four which includes homeostasis, inflammation, proliferation and remodelling. Although the wound healing process is continuous, each phase of wound healing is distinct, with each phase overlapping with the next (Fig. 2.3) (Clark, 1988).

Four phases of wound healing can be described as followed according to (Stadelmann, 1998):

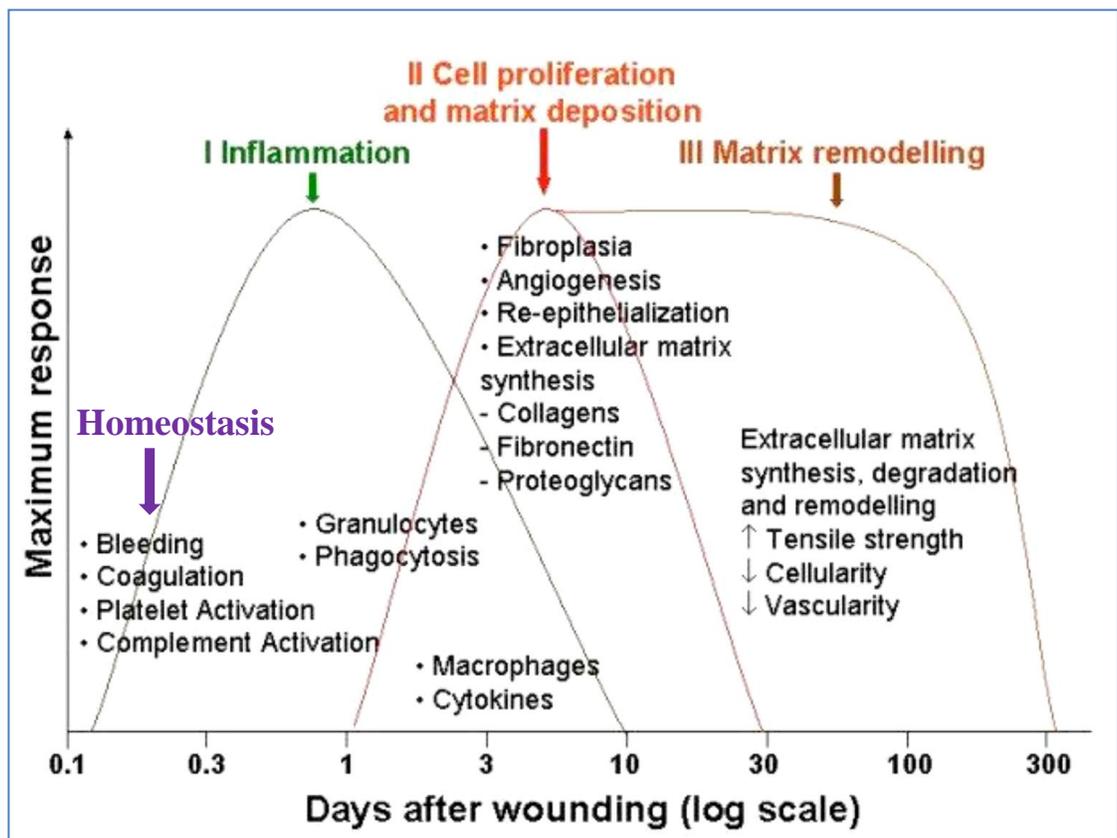


Figure 2.3: Time perspective of wound healing: Phases and processes during healing (Singer & Clark, 1999).

(i) Homeostasis

The initial vascular response to wound is vasoconstriction and homeostasis followed by active vasodilation, and increase in capillary permeability. Damaged blood vessels undergo a reflex vasoconstriction, this is to reduce blood loss and allow the blood time to clot. As a result of hemorrhage the wound fills up with clotted blood. Shortly after the vaso-constrictive phase, release of inflammatory mediators from damaged tissue and mast cells causes an inflammatory vasodilation. The increased flow of red blood cells increases the delivery of oxygen to damaged area, to keep it well oxygenated. This is vital as wound healing is a very energy demanding process. This explains why a good blood supply and effective tissue oxygenation is vital in the process of wound healing. Inflammatory vasodilation has the effect of increasing the physical size of the gaps between adjacent capillary endothelial cells. This promotes increased capillary permeability resulting in them becoming 'leaky'. Increased capillary permeability allows larger molecules, such as fibrinogen, to escape into the tissue spaces. Platelets aggregated within a fibrin clot secrete a variety of growth factors such as transforming growth factor beta and platelet-derived growth factor, and cytokines for tissue repair (Stadelmann, 1998).

(ii) Inflammation

Inflammation presents as erythema, swelling and warmth, and is corresponding with pain. Increase vascular permeability due to the inflammatory response, causes the migration of neutrophils and monocytes into the surrounding wounded tissue. White blood cells are able to migrate from the blood into the tissue spaces; they can squeeze through the enlarged gaps between the capillary endothelial cells. Neutrophils and monocytes arrive via the blood and migrate into the tissue spaces of the wound within the first 24 hours. Once in the tissues, these cells also phagocytose bacteria and dead tissue, this causes them to grow and

they become large cells called macrophages. Neutrophils and macrophages are able to move independently through the tissue spaces using a process called amoeboid movement. Their phagocytic activity is well targeted because they are chemically attracted to bacteria and dead tissue. In addition to phagocytosis, macrophages also coordinate much of the healing process by release of growth factors. The re-growth of epithelium, new capillaries and the migration of fibroblasts stimulate by these locally acting chemicals. At least 20 different growth factors are involved in normal wound healing.

The neutrophils engulf debris and microorganisms, providing the first line of defence against infection. Neutrophils migration ceases after a few days of post injury if the wound is not contaminated. Monocytes are converted in the tissue to macrophages at the late inflammation phase, which digest and kill bacterial pathogens, destroy remaining neutrophils and scavenge tissue debris (Stadelmann, 1998).

(iii) Proliferation

This phase of wound healing starts about 2 to 3 days after the initial injury. It is now necessary for fibroblasts to migrate into the wound where fibroblasts produce the extracellular material needed for fibrous tissue formation. Fibroblasts are attracted into the wound by growth factors released from macrophages and by chemicals released from damaged matrix. Fibroblasts are essential for wound healing; they synthesize and secrete collagen and ground substance. Further growth factors which stimulate and regulate the regeneration of new blood vessels also secreted by fibroblasts, a mechanism called angiogenesis as shown in Fig. 2.4 (Werner & Grose, 2003).

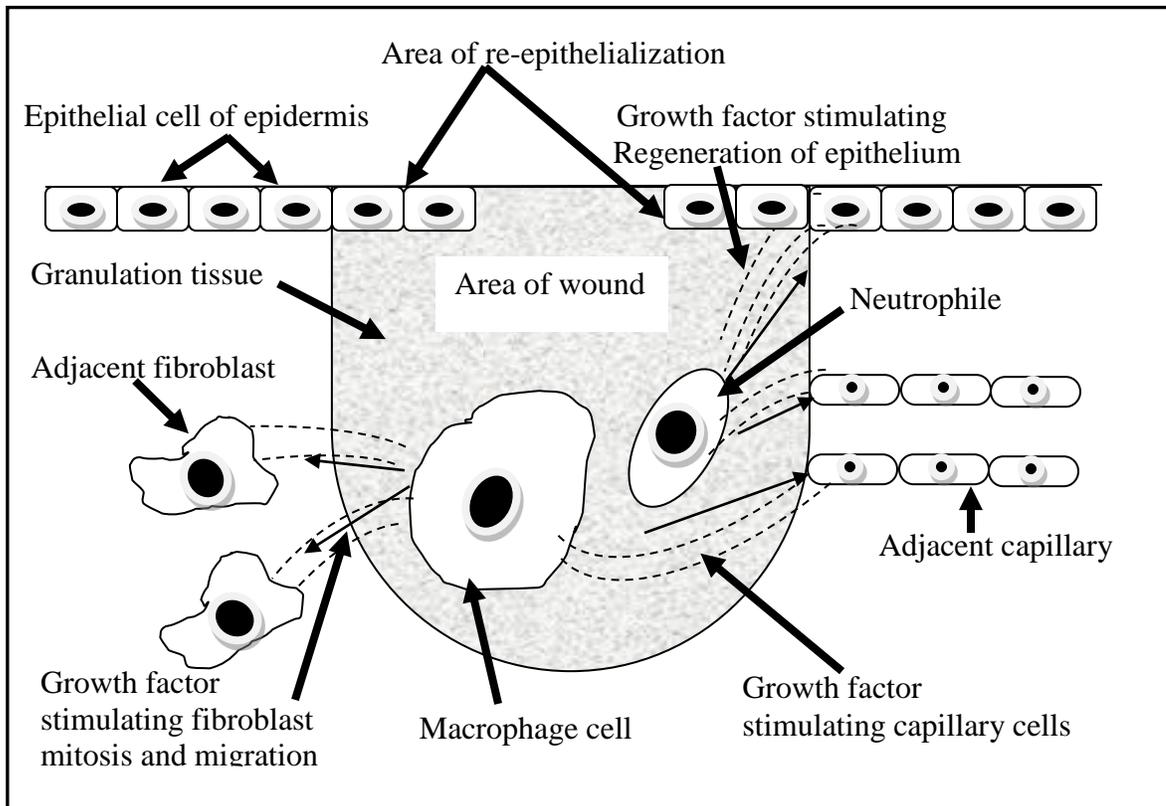


Figure 2.4: Proliferation phase showing cellular stimulation responsible for the processes of fibroblast migration, angiogenesis and re-epithelialization.

Re-epithelialization is part of the proliferation phase. This refers to the re-growth of epithelial tissue. Viable epidermal cells divide by mitosis and start to migrate over the surface of the granulation tissue. Re-epithelialization may develop from the wound edges. Anatomically, epidermis dips down into the hair follicles, into the dermis and even hypodermis. This means there are reserves of epidermal cells in these deeper structures. As a result, the epidermis may regenerate from these preserved deep elements. This means that even when the full thickness of the epidermis is lost, full regeneration is still possible.

Re-epithelialization takes place over the granulation tissue but below the scab on top of the wound, the scab is mostly the residue from the initial blood clot. This scab is very useful as it helps to keep bacteria out of the wound until it can be sealed by the new epithelium. This would kill the new cells and prevent re-epithelialization. Larger wounds

healing by secondary intention also need to have a moist environment to preserve the granulation tissue and promote cellular migration. This is best achieved by using some form of dressing in keeping the natural tissue fluids, while functions these physiological fluids also contain essential growth factors released by macrophages and fibroblasts (Stadelmann, 1998).

(iv) Remodelling

The final phase of wound healing is wound remodelling, including a reorganization of new collagen fibres, thus forming a more structured lattice that progressively continues to increase wound tensile strength. This is also referred to as the maturational phase. It typically begins about 3 weeks after the injury and may go on for a year or more, depending on the size of the wound. Collagen fibers increasingly align themselves with the tensile forces passing through the wound, thus increasing strength. Eventually, the strength of the wound is about 75% that of uninjured tissue. Wound contraction occurs because specialized fibroblasts, called myofibroblasts, join up and contract in a similar way to smooth muscle. Scar vascularity also reduces with time. As vascularity decreases, the scar fades and will eventually become a similar color to the surrounding skin. In some cases of healing wounds where the proliferation of scar tissue continues, resulting in the emergence of a hypertrophic or keloid scar. Hypertrophic scars do not proliferate beyond the limits of the original wound, do not increase in size and often regress after 2-3 months.

The healing of full-thickness skin wounds in adult mammal involves a highly complex and inter-dependent series of repair processes, operating within characteristic spatio-temporal intervals (Clark, 1988). Wound contraction, the biomechanical phenomenon in which the wound boundaries are drawn towards the center is a ubiquitous and essential feature in the healing of these wounds and, together with tissue formation,

normally effects full wound closure (Rudolph, 1980; Rudolph *et al.*, 1992). By reducing the size of the defect, wound contraction is usually beneficial to the overall repair process. However, insufficient contraction may cause delayed or impaired healing, whilst excessive contraction often induces poor quality repair with substantial scarring (Rudolph *et al.*, 1992; Shah *et al.*, 1992). This impaired wound healing in diabetes mellitus is a major clinical problem. The reasons underlying this abnormal wound healing are complicated and remain unclear. Various attempts have been made to accelerate wound healing in diabetes but so far few effective curative remedies are available.

A variety of wound models have been employed to study the wound healing process. The techniques that have been employed involves morphological examination of the wound size, histological examination of biopsied tissue samples, the detection of collagen content, the number of cells in the new connective tissue and epithelial layers (Buffoni *et al.*, 1993) and the measurement of some biochemical parameters (Nangia & Hung, 1990). In conjunction with the area measuring technique and histological examination, evaluation of the effect of mushroom on the proliferation and migration of fibroblasts in culture was included (Su *et al.*, 1999).

2.2.1 Common Types of Wound

i) Contusion

A contusion is more commonly called a bruise. It is usually caused by a blunt blow, the overlying skin is unbroken, but tissues and blood vessels below are damaged. The discolouration is caused by bleeding from small vessels into the tissues. Red blood cells trapped in the tissue spaces become deoxygenated and darkened in color. If deeper tissues, such as nerves, blood vessels or tendons are damaged, bruising can be developed and became apparent after a period of time as blood tracks towards the body surface. If blood

collects in a discrete pool within the tissues, this is described as a haematoma. As the blood in a haematoma is well integrated, it may cause pressure effects on surrounding tissues, which may include pain and nerve compression. There is a risk a haematoma may become infected and some need to be surgically evacuated. Figure 2.5 demonstrates how a contusion or bruise can occur (Campbell, 2009).

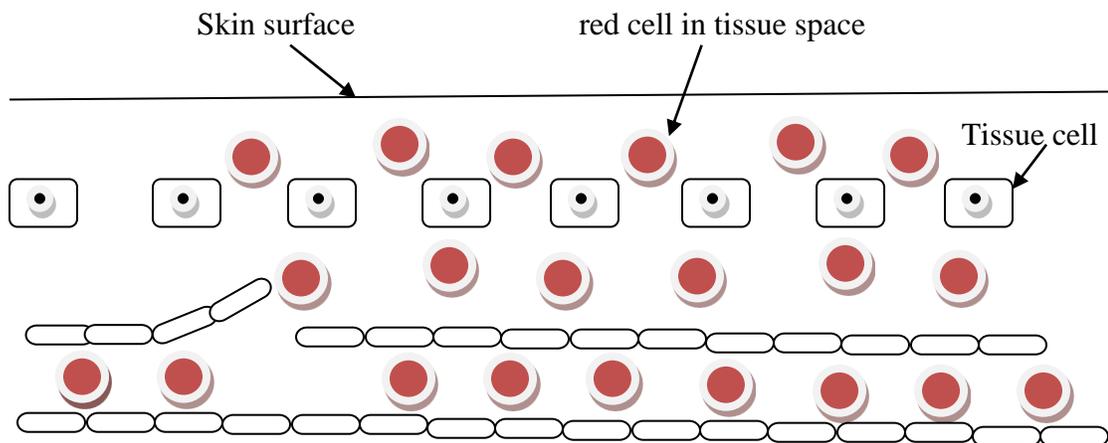


Figure 2.5: A contusion or bruise represents the presence of blood cells in the tissue spaces. This causes a characteristic discolouration of the area. Initially a bruise is ‘black and blue’ due to the presence of reduced haemoglobin in the tissues. Over time macrophages phagocytose the red cells in the tissues and the haemoglobin is converted to bilirubin. This is why the discolouration changes from blue to yellow as the bruise fades.

(ii) Abrasion

An abrasion is a scrape or graze. Typically, there is a superficial surface wound involving the epidermis and part of the dermis. As dermal nociceptors are exposed in the injured dermis, these wounds are frequently very painful. Some abrasions can however, be deeper wounds involving tissues below the level of the skin. Abrasions are most commonly caused by friction injuries or falling off bikes. These wounds need to be well cleaned to remove dirt and grit which may be sticking to the wound surface (Campbell, 2009).

(iii) Avulsion

This term describes a wound where there is tissue loss, preventing the closure of the wound edges. An avulsion may be caused by gouging or tearing of tissue. With an avulsion, a piece of skin is torn loose and is hanging from the body or completely removed. This type of wound can bleed heavily. Avulsions most often involve ears, fingers, and hands (Campbell, 2009).

(iv) Laceration

Laceration describes a wound made by a blunt object, and has often involved considerable force. The wound boundaries are usually split or torn with ragged edges as the skin has been burst rather than cut. After significant trauma, there may be lacerations involving internal organs. Lacerations or tear of the kidneys, liver, or spleen may be associated with serious hemorrhage requiring urgent surgical attention. This is why traumatised patients should be nursed as still as possible, as movement may dislodge blood clots and result in more serious internal haemorrhage (Campbell, 2009).

(v) Incised wound

This is a cut caused by a sharp object. These wounds usually appear neat and the edges can be readily approximated to allow primary healing to take place. In incised wounds the cut may also involve deeper structures such as nerves, blood vessels or tendons. Incised wounds should always be assessed for such deeper injuries and treated as required (Campbell, 2009).

(vi) Puncture wounds

These may well present as misleadingly small wounds and are also described as penetrating wounds. They are made using pointed or sharp objects. As the edges of the

wound may be closed above areas of bacterial contamination, infection is a potential hazard. Moreover, puncture wounds may penetrate down into body cavities or other significant structures such as blood vessels. If the base of a wound cannot be seen it should be surgically assessed as a matter of urgency (Campbell, 2009).

(vii) Strains

Strains are injuries to muscles, tendons or fascia caused by stretching forces. Patients complain of pain and stiffness and there may be some associated swelling. It is usually important to exclude other injuries such as fractures. Strain injuries usually resolve with rest followed by progressive mobilization (Campbell, 2009).

(viii) Sprains

A sprain describes an injury to the fibrous tissues surrounding a joint. Fibrous ligaments around the joint are damaged, usually due to an excessive movement of the joint. A mild sprain may involve tearing a few of the fibres in a ligament, in more serious cases there will be associated haematoma formation. In severe cases there may be complete tearing and disruption of a ligament. Patients usually present with local heat, swelling, pain, disability and possible discoloration over the area. The common sprain is the ankles; if the ankle is turned inwards there will be injury to the lateral ligaments. Sprains usually take longer to recover than strains (Campbell, 2009).

2.2.2 Factors that Influence Wound Healing

Local and systemic factors may influence the rate of wound healing. Local factors describe the conditions in the immediate wound environment while systemic factors refer to ‘whole body’ influences on the local wounded area. Therefore wound healing can be slowed when the patient is diabetic. A crucial point to take into account about a diabetic

patient's wound is that it heals slowly and can worsen rapidly, so close monitoring is mandatory. There are several factors that influence wound healing in a diabetic patient, and these may include:

(i) Blood glucose levels

An elevated blood glucose level stiffens the arteries and causes narrowing of the blood vessels. These affect the origin of wounds as well as risk factors to proper wound healing. It is a common observation that people with diabetes mellitus often have poor wound healing. Unfavorable effects on wound healing are associated to poor glycaemic control. Higher blood glucose levels inhibit wound healing. Reasons for this include high levels of glucose in the tissue fluids and basement membrane thickening in arterioles, capillaries and venous (Campbell, 2009).

(ii) Poor blood circulation

Narrowed blood vessels lead to decreased blood flow and oxygen to a wound. An elevated blood glucose level will decrease the function of red blood cells that carry nutrients to the tissue. It also lowers the efficiency of the white blood cells that fight against infection. Without adequate nutrients and oxygen, a wound heals slowly. Good blood supply to a wound is one of the main factors promoting healing. Wounds on areas of the body with copious blood supplies, such as the face or scalp, tend to heal rapidly. Conversely areas of the body with a deficient blood supply, such as the back or feet, heal more slowly. Blood supply leucocytes, nutrients, oxygen, removes waste products, and keeps the wound warm; all factors which promotes healing. Wound ischaemia may occur as a result of the initial trauma, if blood vessels are damaged or compressed by swelling. Pre-existing vascular insufficiency is a significant adverse factor in healing. Venous deficiency is an adverse factor in wound healing, as seen in venous leg ulceration. Systemic conditions

affecting the cardiovascular system may also reduce local wound perfusion; these may include heart failure or shock. Immobility will also reduce the circulation of the blood and also reduce wound perfusion (Campbell, 2009).

(iii) Diabetic neuropathy

When blood glucose levels are uncontrolled, nerves in patient's body will be affected, causes a loss of sensation. This is called diabetic neuropathy. Due to diabetic neuropathy, patients cannot feel a developing blister, infection or surgical wound problem. The severity of wound can progress and causes complications in healing (Campbell, 2009).

(iv) Delayed inflammatory response

The inflammatory response may be delayed for local or systemic reasons. If the area is cold, there will not be significant inflammation as the vasoconstricting reaction to cold will act against the vasodilatory effect of the inflammatory process. A reduced inflammatory response is also seen in patients who are receiving corticosteroids as these drugs are very anti-inflammatory. Corticosteroids work by decreasing capillary permeability and inhibiting fibroblast activity and the phagocytic capacity of leucocytes. As discussed above, inflammation is the first essential stage in the physiology of wound healing so any factor which reduces this response will delay wound healing (Campbell, 2009).

(v) Infection

It is common observation that infected wounds heal slowly. Infection means that bacteria are present in the wound and are generating an inflammatory host response. The living bacteria produce waste products of their metabolism referred to as exotoxins. These materials are toxic, and so prevent the normal function of local cells and tissues. They may

get involved in protein synthesis. Infected wounds need to be well cleaned and often systemic antibiotics are needed. Any foreign bodies in a wound are also likely to be associated with infection. With a poorly functioning immune system, diabetics are at a higher risk to pick up an infection. Infection increases many health concerns and also slows the overall healing process. Left untreated, infection can heighten the risk of sepsis or a bone infection like osteomyelitis, and developing gangrene. According to statistics, diabetes is the number one reason for limb amputation in the U.S. (Campbell, 2009).

(vi) Oedema

The presence of oedema, for whatever reason, adversely affects wound healing. All cells of the body receive nutrients and oxygen from the capillary blood, via tissue fluid, by the process of diffusion. If there is a raised volume of tissue fluid, as is the case in oedema, then there will be an increase in the distance from the capillaries to the tissue cells. This extended distance means nutrients and oxygen has further to travel to reach the cells, so supplies are reduced. If cell function is reduced wound healing will be correspondingly adversely affected (Campbell, 2009).

2.2.3 Wound Management

The objective of wound management is to heal the wound in the shortest time possible, and ensure that the patient undergo a minimal pain, discomfort, and scarring. At the site of wound closure a flexible and fine scar with high tensile strength is desired for patients. Understanding the healing process and nutritional influences on wound outcome is critical to successful management of wound in patients. Researchers who have explored the complex dynamics of tissue repair have identified several nutritional cofactors involved in tissue regeneration comprises vitamins A, C, and E, arginine, glutamine, glucosamine and

zinc (Mackay & Miller, 2003). Enzyme bromelain from pineapple and botanical extracts from *Aloe vera*, and *Centella asiatica* have also been shown to improve healing time and wound outcome due to their vitamins. Eclectic therapies, including topical application of honey, sugar, comfrey poultices, sugar paste, or *Calendula succus* to open wounds, and hydrotherapy to closed wounds are still in use today (Mackay & Miller, 2003). While anecdotal reports support the efficacy of these eclectic therapies, scientific evidence is lacking. Other factors that may influence wound healing include adequate tissue perfusion, blood flow, and oxygen levels in the wound tissue. The synthesis of the enzymatic hydroxylation of proline and lysine residues and fibroblasts on the forming collagen chains are dependent, in part, on the availability of oxygen (Whitney & Heitkemper, 1999). Finally, it is important to remember that a wound dressing does not heal the wound but it provides the optimum environment for healing to take place.

2.3 Mushrooms in Wound Healing

Plants and fungi are traditionally used for the treatment of diverse ailments in mankind since ancient time. Least but not less they are also been studied for their anti-inflammatory, antioxidant, and immune-modulating effects associated with wound healing potential. The pharmacological studies focused on the wound healing promoting effect of mushroom's polysaccharides are rather scarce.

Bae *et al.*, (2005) reported that the polysaccharides isolated from *Phellinus gilvus* (Schw.) Patouillar (mustard-yellow polypore) enhanced dermal wound healing in normal and streptozotocin-induced diabetic rats. Kwon *et al.*, (2009) also reported that the oral administration of β -glucan purified from medicinal mushroom *Sparassis crispa* (cauliflower mushroom) at 40%, increased macrophage infiltration into the wound tissue and enhanced wound healing. So, the mechanism of β -glucan-induced wound healing was

associated with increased type I and III collagen biosynthesis (Kwon *et al.*, 2009). Most recently, polysaccharides purified from *Tremella fuciformis* (white jelly mushroom) and *Auricularia auricula* (wood ear mushroom) were shown to enhance wound healing using the *ex vivo* porcine skin wound healing model (Khamlue *et al.*, 2012).

Furthermore, medicinal mushroom also been proven to reduce ulceration in ethanol-induced gastric ulcer. Oral feeding of *Lentinus squarrosulus* extract (250mg) offered significant gastric mucosal protection of *Sprague-Dowley* rats comparable to cimetidine (50mg/kg) (Omar *et al.*, 2011). Accordingly, the ulcer healing rate in rats after 24, 48 and 72 hours of treatment was at 82%, 90% and 100% respectively. The results of IL-1 beta level in serum and the NF-Kappa B levels in tissue homogenate indicated that the healing potential was associated with attenuation proinflammatory cytokines (Mohamad Omar *et al.*, 2011). *Hericium erinaceus* (Bull.:Fr.) Pers. (Aphylophoromycetidae) has shown to accelerate wound healing in rats, which reduced scar width at wound closure and healed wound containing more collagen, angiogenesis compared to wounds dressed with distilled water (Abdulla *et al.*, 2011). The same mushroom also has been found to reduce ulceration in ethanol-induced gastric ulcers in rats (Abdulla *et al.*, 2008). Administration of *Lentinus edodes* polysaccharides significantly raised activities of serum antioxidant enzyme and decreased levels of serum mucosal interleukin-2 (IL-2) and TNF- α in rats with oral ulceration (Yu *et al.*, 2009c).

Elsewhere, Sun *et al.*, (2011) reported that *G. lucidum* polysaccharides showed wound healing effects on intestinal epithelium using a non-transformed small-intestinal epithelial cell line, Intestine Epithelioid Cell Line-6 cells. Sacchachitin membrane (trade name Beschitin W) prepared from residue of fruiting bodies of *G. tsugae* after aqueous extraction was found to have wound healing properties similar to that of chitin from crab shell (Su *et al.*, 1997). Polysaccharides fractions from *G. lucidum* have been

shown to have healing effect on acetic acid-induced ulcers in rats, which demonstrated that *G. lucidum* could be a useful preparation on the prevention and treatment of peptic ulcers (Gao *et al.*, 2004). Despite all the beneficial wound-healing effects of medicinal mushrooms, little attention has been paid to aqueous extracts of *G. lucidum* on wounds associated to diabetes.

2.4 *Ganoderma lucidum*

Ganoderma lucidum, an oriental fungus has a long history of use for promoting health and longevity in China, Japan, and other Asian countries. It is a large, yellow to dark brown mushroom with a glossy exterior and a woody texture. The Latin word *lucidus* means “shiny” or “brilliant” and refers to the varnished appearance of the surface of the mushroom. The appearance of the fruiting bodies of *Ganoderma lucidum* is illustrated in Plate 2.2.



Plate 2.2: Fruiting bodies of *Ganoderma lucidum* (Ganofarm Sendirian Berhad, date: 23rd October 2013).

In Japan the name for the Ganodermataceae family is reishi or mannentake, whereas in China, *G. lucidum* is called lingzhi. In Chinese, the name lingzhi represents a combination of spiritual potency and essence of immortality, and is regarded as the “herb of spiritual potency,” symbolizing divine power, success, well-being, and longevity. *G.*

lucidum is unique in that its pharmaceutical rather than nutritional value is paramount compared to other cultivated mushrooms. The specific applications and attributed health benefits of lingzhi include control of modulation of the immune system, blood glucose levels, bacteriostasis, hepatoprotection, and more. The various credence regarding the health benefits of *G. lucidum* are based largely on anecdotal evidence, traditional use, and cultural mores. However, recent reports provide scientific support to some of the ancient claims of the health benefits of Ganoderma.

2.4.1 History of *G. lucidum*

G. lucidum has been recognized as a medicinal mushroom for more than 2000 years, and its intoxicating effects have been documented in ancient scripts (Wasser *et al.*, 2005). The proliferation of *G. lucidum* images in art began in 1400 AD, and they are associated with Taoism (McMeekin, 2005). However, *G. lucidum* images extended beyond religion and appeared in paintings, furniture, carvings, and even women's accessories (Wasser, 2005). The first book wholly devoted to the description of herbs and their medicinal value was written in the Eastern Han dynasty of China (25-220 AD) by *Shen Nong Ben Cao Jing*. This book is also known as "The Classic of the Materia Medica" or "Shen-nong's Herbal Classics." It was composed in the second century under the pseudonym of Shen-nong, discusses zoological, botanical, and mineral substances ("the holy farmer"; Zhu, 1998). The book describes the beneficial effects of several mushrooms with a reference to *G. lucidum* and have been continually updated and extended (Zhu, 1998). In the *Supplement to Classic of Materia Medica* (502-536 AD) and the *Ben Cao Gang Mu* by Li Shin-Zhen, a book considered to be the first pharmacopoeia in China (1590 AD; Ming dynasty), the mushroom have therapeutic properties, such as enhancing vital energy, tonifying effects, increasing memory, strengthening cardiac function, and anti-

aging effects. Based on the *State Pharmacopoeia of the People's Republic of China* (2000), *G. lucidum* acts to replenish Qi, ease the mind, and relieve cough and asthma, and it is recommended for insomnia, dizziness, shortness of breath and palpitation. Nevertheless, the *Ganoderma* species continue to be a popular traditional medicine in Asia and their use is growing throughout the world (Wachtel-Galor *et al.*, 2004).

2.4.2 Taxonomy

The family Ganodermataceae describes polypore basidiomycetous fungi having a double walled basidiospore (Donk, 1964). In all, 219 species within the family have been assigned to the genus *Ganoderma*, of which *G. lucidum* (W. Curt.: Fr.) P. Karsten is the species type (Moncalvo, 2000). Basidiocarps of this genus have a laccate (shiny) surface that is associated with the presence of thick-walled pilocystidia embedded in an extracellular melanin matrix (Moncalvo, 2000). *Ganoderma* species are found all over the world. Dissimilar characteristics, such as shape and color (white, red, black, blue/green, yellow, and purple) of the fruit body, geographical origin, and host specificity, are used to identify individual members of the species.

Unfortunately, the morphological characteristics are subject to variation; for example, due to differences in cultivation in different geographical locations under different climatic conditions and the natural genetic development (e.g., mutation, recombination) of individual species (Upton, 2000). Consequently, the use of macroscopic characteristics has resulted in a large number of synonyms and overlapping, and unclear taxonomy for this mushroom. More reliable morphological characteristics for *Ganoderma* species are thought to include context color and consistency, spore size and shape, and the microanatomy of the pilear crust. Other characteristics have also been used for differentiating morphologically similar species such as chlamydospore production and shape, enzymatic studies and, to a

lesser extent, the range and optima of growth temperatures (Moncalvo, 2000). Biochemical, genetic, and molecular approaches have also been used in *Ganoderma* species taxonomy.

2.4.3 Cultivation

Attempts were made to cultivate the mushroom due to its irregular distribution in the wild and increasing demand as medicinal herb (Chang & Buswell, 2008). Cultivation of *G. lucidum* has become a great source of the mushroom since the early 1970s. Artificial cultivation of *G. lucidum* has been achieved using substrates such as grain, sawdust, wood logs (Chang & Buswell, 1999; Wasser, 2005). *G. lucidum* is a well-known Asian herbal remedy with a wide range of applications. Consumption of *G. lucidum* in global is high, and a massive, increasing series of patented and commercially available products that include *G. lucidum* as an active ingredient are obtainable as food supplements. These incorporate extracts and isolated constituents in various formulations, in the form of capsules, creams, hair tonics, and syrups which are marketed all over the world (Wachtel-Galor, *et al.*, 2011).

More than 90 brands of *G. lucidum* products were registered and marketed internationally a decade ago (Lin, 2000). There are several thousand tonnes worldwide consumption is now estimated and the market is growing rapidly. Despite no recently published data relating to the total world market value of *Ganoderma* products; in 1995 the total approximated annual market value given by different commercial sources was US\$1628 million (Chang & Buswell, 1999). Numerous products, prepared from dissimilar parts of the mushroom, are currently available on the market (Chang & Buswell, 2008).

2.4.4 Major Bioactive Components

Most mushrooms are composed of around 90% water by weight. The remaining 10% consists of 10–40% protein, 2–8% fat, 3–28% carbohydrate, 3–32% fibre, 8–10% ash, and some vitamins and minerals, with calcium, potassium, phosphorus, magnesium, selenium, iron, copper and zinc, accounting for most of the mineral content (Borchers *et al.*, 1999). In a study of the non-volatile elements of *G. lucidum*, shows that the mushroom contains 1.8% ash, 3–5% crude fat, 7–8% crude protein, 26–28% carbohydrate, and 59% crude fibre (Mau *et al.*, 2001). In addition to these, mushrooms contain a wide variety of bioactive molecules, such as steroids, terpenoids, nucleotides, phenols, and their derivatives, polysaccharides and glycoproteins. Mushroom proteins carry all the essential amino acids and are especially rich in leucine and lysine. The low overall fat content and high percentage of polyunsaturated fatty acids relative to the total fatty acids of mushrooms are considered significant contributors to the health value of mushrooms (Chang & Buswell, 1996; Borchers *et al.*, 1999).

2.4.4.1 Polysaccharides and Peptidoglycans

Peptidoglycans, polysaccharides, and triterpenes are three primary physiologically active constituents in *G. lucidum* (Boh *et al.*, 2007; Zhou *et al.*, 2007). However, the quantity and percentage of each component can be very diverse in natural and commercial products. Fungi are remarkable for the variety of high-molecular-weight polysaccharide structures they generated, and bioactive polyglycans are found in all parts of the mushroom.

Polysaccharides represent multiple biological macromolecules structurally, with wide-ranging physiochemical properties (Zhou *et al.*, 2007). Various polysaccharides have been extracted from the fruit body, mycelia and spores of lingzhi; they are produced by fungal mycelia cultured in fermenter and can differ in their sugar and peptide compositions

and molecular weight (e.g., ganoderans A, B, and C).

G. lucidum polysaccharides (GL-PSs) are reported to exhibit a broad range of bioactivities, including antiulcer, hypoglycemic, immunostimulating, anti-inflammatory, and antitumorigenic effects (Wachtel-Galor *et al.*, 2004). Polysaccharides are normally obtained from the mushroom by extraction with hot water followed by precipitation with methanol or ethanol, but also can be extracted with alkali and water. Structural analyses of GL-PSs specify that glucose is their major sugar component (Bao *et al.*, 2001; Wang *et al.*, 2002). Anyway, GL-PSs are heteropolymers and can also contain galactose, xylose, mannose, and fuctose in different conformations, including 1–3, 1–4, and 1–6-linked β and α -D (or L)-substitutions (Bao *et al.*, 2002). Branching conformation and solubility characteristics are said to affect the antitumorigenic properties of these polysaccharides (Bao *et al.*, 2001; Zhang *et al.*, 2001). The mushroom also consists of a matrix of the polysaccharide chitin, which is mainly indigestible by human body and is partly responsible for the physical hardness of the mushroom (Upton, 2000). Numerous refined polysaccharide preparations extracted from *G. lucidum* are now sold as over-the-counter treatment for chronic diseases, including cancer and liver disease (Gao *et al.*, 2005).

Various bioactive peptidoglycans have also been isolated from *G. lucidum*, including, F3 (a fucose-containing glycoprotein fraction; Chien *et al.*, 2004); *G. lucidum* proteoglycan (GLPG; with antiviral activity (Li *et al.*, 2005); *G. lucidum* immunomodulating substance (GLIS; Ji *et al.*, 2007), PGY (a water-soluble glycopeptide fractionated and purified from aqueous extracts of *G. lucidum* fruit bodies (Wu & Wang, 2009), and GL-PS peptide (GL-PP; Ho *et al.*, 2007).

2.4.4.2 Triterpenes

Terpenes are a class of naturally occurring compounds whose carbon skeletons are composed of one or more isoprene C₅ units. Examples of terpenes are menthol (monoterpene) and β-carotene (tetraterpene). Numerous are alkenes, although some carry other functional groups, and many are cyclic. Triterpenes are a subclass of terpenes and have a basic skeleton of C₃₀. In general, triterpenoids have molecular weights ranging from 400 to 600 kDa and their chemical structure is complex and highly (Zhou *et al.*, 2007).

During the past two decades, more than 130 triterpenes (including ganoderic acid derivatives) have been isolated from the fruiting bodies, cultured mycelia and spores of the *Ganoderma* (Luo *et al.*, 2002). Among them, more than 50 were found to be new and unique to this fungus. The most are lucidenic and ganoderic acids, but other triterpenes such as ganoderals, ganodermic and ganoderiols acids have also been identified (Zhou *et al.*, 2007; Chen *et al.*, 2010). Triterpenes have received considerable attention owing to their conspicuous pharmacological activities. Some of these compounds showed anti-HIV-1 (ganoderic acids A, B, H, C1) (Mekkawy *et al.*, 1998), antihistamine (ganoderic acids C2, D) (Kohda *et al.*, 1985), anti-cholesterol (ganoderic acids B, C2) (Komada *et al.*, 1989), and inhibitory activity of angiotensin converting enzyme (ganoderic acids K, F, S) (Morigiwa *et al.*, 1986). Hence, the triterpenoids could be considered as the ‘marker compounds’ for the chemical evaluation or standardization of *Ganoderma* spp.

Ganoderma lucidum is clearly rich in triterpenes, which gives the herb its bitter taste and, it is believed, endow with it various health advantages such as antioxidant effects and lipid-lowering. However, the triterpenes content is different in different parts and growing stages of the mushroom. The form of dissimilar triterpenes in *G. lucidum* can be used to differentiate this medicinal fungus from other taxonomically related species, and can act as supporting evidence for classification. The triterpenes content can also be used as

a measure of quality of different ganoderma samples (Chen *et al.*, 1999; Su *et al.*, 2001).

2.4.4.3 Other Components

Elemental analysis of log-cultivated fruit bodies of *G. lucidum* revealed potassium, phosphorus, silica, sulphur, magnesium and calcium to be their major mineral components. Sodium, zinc, iron, copper, strontium, and manganese were also detected in lower quantity, as well as heavy metals lead, mercury and cadmium (Chen *et al.*, 1998). Freeze-dried fruit bodies of unidentified wild *Ganoderma* spp. were reported to have a mineral content of 10.2%, with potassium, calcium, and magnesium as the major components (Chiu *et al.*, 2000).

Some attention has been given to the germanium content of *Ganoderma* spp. Among the minerals detected in *G. lucidum* fruit bodies collected from the wild, germanium was fifth highest in terms of concentration (489 µg/g) (Chiu *et al.*, 2000). This mineral is also present in the order of parts per billion in many plant-based foods, including ginseng, aloe, and garlic (Chiu *et al.*, 2000). Although germanium is not an important component, at low dosage it has been credited with immunopotentiating, antioxidant, antitumor, and antimutagenic activities (Kolesnikova *et al.*, 1997). However, although the germanium content of *G. lucidum* has been used to stimulate *G. lucidum*-based products, there is no solid evidence linking this element with the specific health benefits associated with the mushroom.

Ganoderma lucidum contain compounds such as lectins and proteins that may contribute to its medicinal effect. Protein content of dried *G. lucidum* is around 7–8%, lower than that of many other mushrooms (Mau *et al.*, 2001). Bioactive proteins including LZ-8, an immunosuppressive protein purified from the mycelia are reported to contribute to the medicinal properties of *G. lucidum* (Van Der Hem *et al.*, 1995); a peptide preparation

(GLP) exhibiting hepatoprotective and antioxidant activities (Shi, *et al.*, 2008); and ganodermin, a 15-kDa antifungal protein isolated from *G. lucidum* fruiting bodies (Wang & Ng, 2006). Lectins, including a novel 114-kDa hexameric lectin; a glycoprotein having 9.3% neutral sugar and showing hemagglutinating activity on pronase-treated human erythrocytes were also isolated from the fruit body and mycelium of the mushroom (Thakur *et al.*, 2007).

Other compounds such as metalloprotease have been isolated from *G. lucidum* include enzymes which delays clotting time; ergosterol (provitamin D₂); nucleosides; and nucleotides (adenosine and guanosine; Wasser *et al.*, 2005; Paterson, 2006). Kim and Nho (2004) also described the isolation and physicochemical properties of a highly specific and effective reversible inhibitor of α -glucosidase, saccharomyces genome protein of unknown function (SKG-3), from *G. lucidum* fruiting bodies. Furthermore, *G. lucidum* spores were reported to contain a mixture of several long-chain fatty acids that may contribute to the antitumor activity of the mushroom (Fukuzawa *et al.*, 2008).

2.4.5 Therapeutic Applications of *Ganoderma lucidum*

For hundreds of years, this mushroom has been regarded as a traditional Chinese medicine or a folk medicine used for the prevention and treatment diseases, such as immunological disorders, hypertension, chronic bronchitis, hepatitis, tumorigenic disease, and hypercholesterolemia in China and other Asian countries (Lin, 2001). The combination of benefit without toxicity represents the desired end result in the development of effective therapeutic interventions. There are now many published studies that are based on animal and cell culture models, on in vitro assessment of the health effects and some reports of human trials in the field. Anyway, there is no cohesive body of research, and the objective analysis of this traditional therapy in terms of human health remains to be clearly

established (Paterson, 2006).

2.4.6 Antioxidant Activities of *Ganoderma lucidum*

Antioxidants protect cellular components from oxidative damage, which is likely to lower risk of carcinogenesis and mutations and also protect immune cells, allowing them to preserve immune surveillance and response. Various elements of *G. lucidum*, in particular triterpenoids and polysaccharides, show antioxidant activity in vitro (Wu & Wang, 2009).

Ooi and Liu (2000) reported that protein-bound polysaccharide and polysaccharide peptide were able to mimic the endogenous antioxidant superoxide dismutase in cancer-bearing animals in vivo. Moreover, these polysaccharides were also reported to protect the immune cells from oxidative damage (Ooi & Lui, 2000). The protective effects of *G. lucidum* on DNA strand scission promoted by an ultraviolet irradiation, metal-catalyzed Fenton reaction, and hydroxyl radical attack were shown in agarose gel electrophoresis in vitro (Lee *et al.*, 2001). Hot water extracts of *G. lucidum* considerably protected Raji cells from hydrogen peroxide (H₂O₂)-induced DNA damage (Shi *et al.*, 2002). Hot water extracts protected human lymphocyte DNA only at low (<0.001% w/v) concentrations, and caused H₂O₂-mediated damage at higher concentrations (>0.01% w/v) (Wachtel-Galor *et al.*, 2005). Two antioxidant-enriched extracts from *G. lucidum* functioned differently in premalignant HUC-PC cells under carcinogenic attack (Yuen & Gohel, 2008). The aqueous extract protected cellular DNA from oxidative damage, while the ethanolic extract destroyed cellular DNA, with raised H₂O₂ production and notable cell-killing effects observed. The results recommended that dissimilar effects of *G. lucidum* could be exhibited by dissimilar extractable components in bladder chemoprevention. *G. lucidum*'s methanol extracts were reported to prevent kidney damage (induced by the anticancer drug cisplatin) through restoration of the renal antioxidant defense system (Sheena *et al.*, 2003). In

contrast, a fraction of ganoderma triterpenes (GTS) was found to enhance the intracellular ROS-producing effect of doxorubicin (DOX) in HeLa cells, which lead to more DNA damage and apoptosis, whereas such cooperative interaction was inhibited by a ROS scavenger (Yue *et al.*, 2008). In an animal study (diabetic rats), non-enzymatic and enzymatic antioxidants levels increased and lipid peroxidation levels decreased with *G. lucidum* treatment (Jia *et al.*, 2009). However, a direct link has not been established between the antioxidant properties of *G. lucidum* and its anticancer and immunomodulatory effects whether lingzhi acts as a pro-oxidant or antioxidant may depend on concentration and environment.

2.4.7 Hypoglycemic Effect of *Ganoderma lucidum*

Components of *G. lucidum* have been proved to have a hypoglycemic effect in animals. The administration of two polysaccharides isolated from fruit-body water extracts, ganoderans A and B (dose of 100 mg/kg), by intraperitoneal injection to normal and alloxan-induced diabetic mice significantly decreased (by up to 50%) the plasma glucose concentrations. In addition, hypoglycemic effect was still evident after 24 hours (Hikino *et al.*, 1985). Using a mouse test sample, ganoderan B was also reported to decrease hepatic glycogen content, increase plasma insulin, and modulate the activity of glucose-metabolizing enzymes in the liver (Hikino *et al.*, 1989). The same group reported that ganoderan B increased plasma insulin levels in both normal and glucose-loaded mice and that a third polysaccharide (ganoderan C) isolated from *G. lucidum* also showed significant hypoglycemic effects in mice (Hikino *et al.*, 1989).

In a more recent study, oral administration of *G. lucidum* hot water extract (0.03 and 0.3 g/kg BW) for 4 weeks was found to lower the serum glucose levels in obese/diabetic (+db/+db) mice, with effects seen after the first week of treatment (Seto *et al.*, 2009).

However, insulin levels were not altered and the glucose levels were still higher in these animals than in the control animals. The extract markedly decreased levels of phosphoenol-pyruvate carboxykinase (PEPCK), which are usually high in obese/diabetic mice. The recommended mechanism, according to the authors, is that of decreasing the serum glucose levels through suppression of the hepatic phosphoenol-pyruvate carboxykinase (PEPCK) gene expression. In another study (Jia *et al.*, 2009), a polysaccharides-rich extract revealed beneficial effects in streptozotocin-induced diabetic rats. The diabetic rats were treated with *G. lucidum* for 30 days. After the treatment, serum insulin levels increased (compared with the non-treated diabetic group) and glucose levels decreased in a dose-dependent way. Similarly, treatment with streptozotocin cause an elevated levels of lipid peroxidation markers (thiobarbituric acid reactive substances [TBARS]), lipid hydroperoxides, and conjugated dienes); decreased levels of non-enzymic antioxidants (vitamin C, reduced glutathione [GSH], vitamin E); and decreased activities of the antioxidant enzymes, catalase, SOD, and glutathione peroxidase (Gpx). Following treatment with GL-PSs, levels of non-enzymatic and enzymatic antioxidants increased and lipid peroxidation levels decreased. Hence, in addition to its glycemic modulation, treatment with *G. lucidum* assisted in decreasing oxidative stress (Jia *et al.*, 2009).

In one study reported in the literature, 71 confirmed Type-2 diabetes mellitus (DM) adult patients were supplemented with Ganopoly (polysaccharide fractions extracted from *G. lucidum*). Three times daily for 12 weeks, the patients received either 1800 mg Ganopoly or placebo, orally. After 12 weeks, Glycosylated hemoglobin (HbA_{1c}) and plasma glucose decreased significantly, indicating a hypoglycemic effect of the extract (Gao *et al.*, 2004). Overall, the data from different studies recommend that *G. lucidum* admission helps in modulating blood glucose levels. Anyway, most studies have been performed on animals or in cell-culture models. Thus, more information from well-

planned human clinical studies is needed with and without combination with conventional medicines (Wachtel-Galor *et al.*, 2011).

With its growing popularity, many studies on *G. lucidum* composition and reputed therapeutic effects are being carried out, and there are data that support its positive health, including good blood glucose regulation, anticancer effects; antiviral effects; antioxidant, and antibacterial; and protection against liver and gastric injury. Currently, the great wealth of chemical data and anecdotal evidence on the effects of *G. lucidum* should be complemented by reliable experimental and clinical data from well-designed human trials in order to clearly establish if the reported health-related effects are valid and significant. Countless challenges are encountered due to a range of factors from dosage to production standard. Strategies for further improve the quality control procedures to define and standardize *G. lucidum* production are needed to determine mechanisms of action and to help characterize the active component(s) of this putative medicinal mushroom (Wachtel-Galor *et al.*, 2011).

2.5 Oxidative Stress and Antioxidant

Oxidative stress is the steady state level of oxidative damage in a cell, tissue, and organ caused by reactive oxygen species. To protect from these highly reactive intermediates, living organisms possess a defence system consisting of both enzymatic and non-enzymatic antioxidants that scavenge them. However, under several situations, the rate of generation of ROS exceeds that of their removal; thus oxidative stress occurs (Halliwell & Gutteridge, 2000). When the stress level exceeds defence capacity, and leads to oxidative damage, the low level stress may stimulate defence network and induce adaptive response (Niki & Yoshida, 2005).

Oxidative stress is defined in general as excess formation and/or insufficient removal of highly reactive molecules such as ROS and reactive nitrogen species (RNS). ROS include free radicals such as superoxide ($\bullet\text{O}^{2-}$), hydroxyl ($\bullet\text{OH}$), peroxy ($\bullet\text{RO}_2$), hydroperoxyl ($\bullet\text{HRO}_2$) as well as non-radical species such as hydrogen peroxide (H_2O_2) and hydrochlorous acid (HOCl) (Maritim *et al.*, 2003). RNS include free radicals like nitric oxide ($\bullet\text{NO}$) and nitrogen dioxide ($\bullet\text{NO}_2$), as well as non-radicals such as peroxynitrite (ONOO^-), nitrous oxide (HNO_2) and alkyl peroxynitrates (RONOO) (Evans *et al.*, 2002).

There are multiple sources of oxidative stress in diabetes including non-enzymatic, enzymatic and mitochondrial pathways. Non-enzymatic sources of oxidative stress originate from the oxidative biochemistry of glucose. Hyperglycemia can directly cause increased ROS generation. The mitochondrial respiratory chain is another source of non-enzymatic generation of reactive species. Reactive species can be eliminated by a number of enzymatic and non-enzymatic antioxidant mechanisms. As discussed above, SOD immediately converts $\bullet\text{O}^{2-}$ to H_2O_2 , which is then detoxified to water either by catalase in peroxisome or by glutathione peroxidase in the mitochondria (Fig. 2.6). Another enzyme that is important is glutathione reductase, which regenerates glutathione that is used as a hydrogen donor by glutathione peroxidase during the elimination of H_2O_2 . Glutathione acts as a direct scavenger as well as a co-substrate for GSH peroxidase. It is a major intracellular redox tampon system (Schultz *et al.*, 2005). Generation of reactive species in diabetes is depicted in Fig. 2.5.

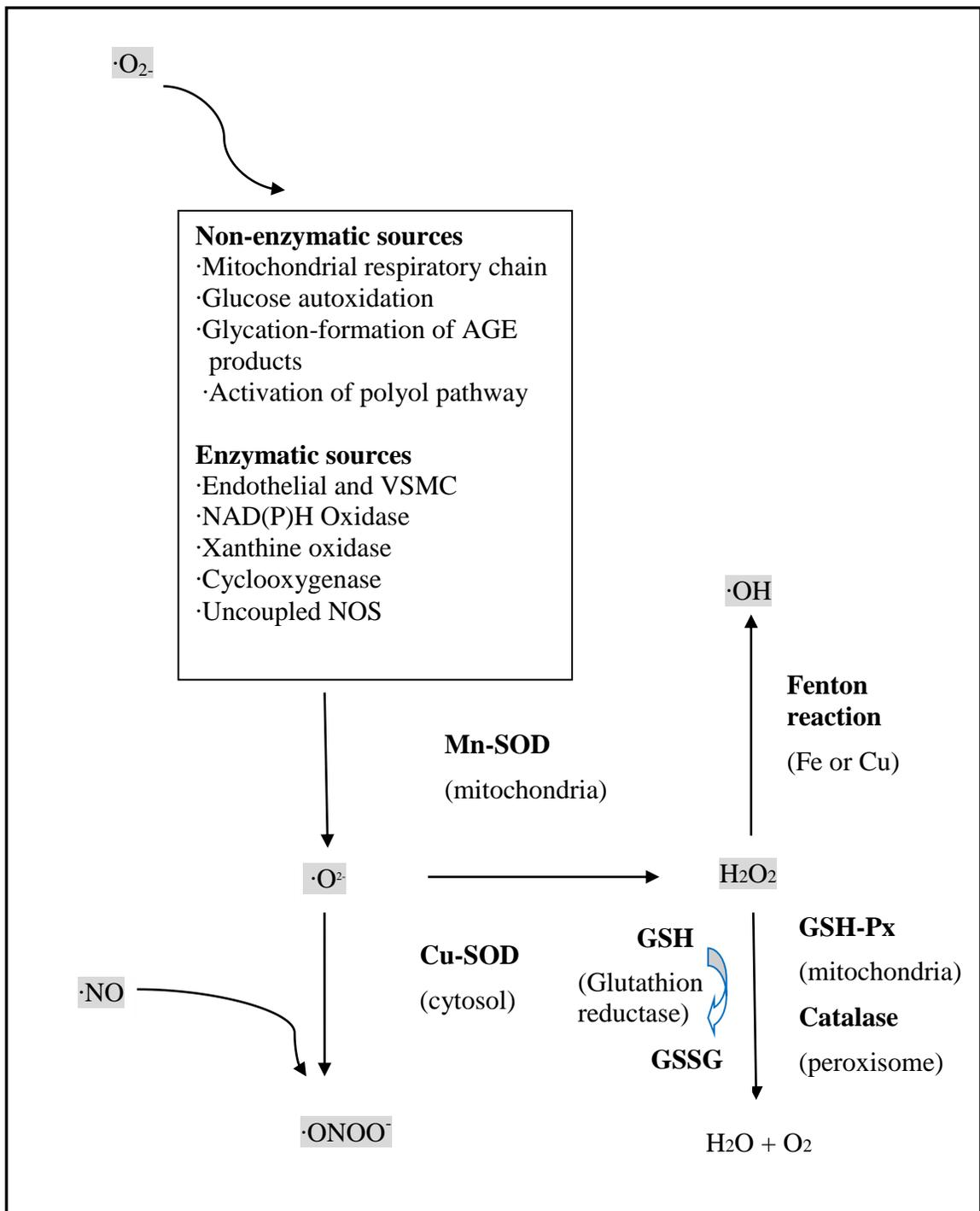


Figure 2.6: Generation of reactive species in diabetes. Highlighted in grey are some of the most important ROS and RNS in vascular cells. Oxygen is converted to $\cdot\text{O}_2^-$ via the activation of enzymatic and non-enzymatic pathways, which is then dismutated to H_2O_2 by SOD. H_2O_2 can be converted to H_2O by catalase or glutathione peroxidase (GSH-Px) or to $\cdot\text{OH}$ after reaction with Cu or Fe. Glutathione reductase regenerates glutathione (GSH). In addition, $\cdot\text{O}_2^-$ reacts rapidly with $\cdot\text{NO}$ to form ONOO^- (Johansen *et al.*, 2005).

The antioxidant defence system of the body consists of endogenous and exogenous antioxidants that work together at the molecular level to protect lipoproteins, cell membranes, and DNA from the damaging effects of free oxygen radicals (Halliwell *et al.*, 1990). However, the capability of such protective systems gradually decreases with age, resulting in disturbances in the normal redox equilibrium that is established in healthy systems (Ramarathnam *et al.*, 1995). Glutathione acts as a major antioxidant buffer within the cell. Moreover, several enzymatic systems detoxify ROS: catalase dismutates H_2O_2 and superoxide dismutase eliminates O^{2-} (but generates H_2O_2). Glutathione peroxidase catalyzes the reduction of peroxides (hydroperoxides, including H_2O_2) into alcohols by using the reducing potential of glutathione. In addition to these endogenous ROS scavengers, the body can be supplied with exogenous antioxidants such as vitamin E, vitamin C and carotenoids. At least some essential trace elements (selenium, copper, zinc and manganese) and other compounds such as flavonoids, sulfur-containing compounds, amino acids and several phytochemicals contribute to the anti-oxidative defence as cofactors to a greater or lesser extent (Biesalski, 1995).

Factors that may contribute to the failure of some wounds to heal include elevated levels of oxygen free radicals, products of oxidation which chemically alter the essential components in cell (James *et al.*, 2003; Moseley *et al.*, 2004). The ROS play an important role related to the pathological processes of various diseases, such as hyperglycemic and impaired wound healing (Domenica *et al.*, 2001).

Overproduction of ROS results in oxidative stress thereby causing cytotoxicity and delayed wound healing (Dissemond *et al.*, 2002). Therefore, estimation of ROS by antioxidants like catalase, SOD, and glutathione in granulation tissues is also relevant because these antioxidants hasten the process of wound healing by destroying the free radicals (Halliwell *et al.*, 1988). Gupta *et al.*, (2002) reported that the significant alteration

in the antioxidant profile accompanied by the increased malondialdehyde (MDA) level a marker of free radical damage may be accredited to impaired wound healing in immune compromised rats. Since numerous studies demonstrated that oxidative stress, mediated primarily by hyperglycemia-induced generation of free radicals, leads to the development and progression of diabetes and related contributions. Ameliorating oxidative stress through treatment with antioxidants exogenously or endogenously might be an effective strategy for reducing diabetic complications (Johansen *et al.*, 2005).

The antioxidants present in mushrooms are of great interest as protective agents help the human body to reduce oxidative damage without any interference. They are recognized as functional foods and as source of physiologically beneficial components and are considered to be a good source of phenolic antioxidants, such as diboviquinone and variegatic acid (Wasser *et al.*, 1999). Mushrooms also have powerful antioxidant properties derived from compounds such as selenium, ergothioneine and phenolics. Selenium is a component of the antioxidant enzyme selenoproteins. Ergothioneine is a thiol compound and natural antioxidant of biological origin. It has been implicated in the intracellular antioxidant defence system along with the prototypic water-soluble thiol antioxidant glutathione. It may be useful to serve as antioxidant agent and free radicals scavenger in enhancing chronic wound healing in diabetic condition (Harshman & Aldoori, 2005).

2.5.1 Oxidation biomarker

Oxidative stress is involved in various disorders and diseases, and received much attention. Biomarkers are important measure to assess the extent of oxidative stress *in vivo*. Several biomarkers have been used, such as AOPP (Škvařilová *et al.*, 2005), LHP (Asano *et al.*, 2009), thiobarbituric acid reactive substances (TBARS), ethane and pentane in exhaled gas, protein carbonyl, aldehyde-modified proteins, and 8-oxodeoxyguanosine (Niki *et al.*, 2005).

Impaired diabetic wound healing constitutes a serious diabetic complication with increased mortality, morbidity, and health expenditure. The exact pathogenetic process has not been totally clarified. A variety of hyperglycemia and oxidative stress related factor, have been proposed, including advanced glycation end products (AGE), advanced oxidative protein products (AOPP). Many studies have shown that lipid hydro-peroxides and reactive oxygen species are involved in the development of a variety of diseases, including cancer, diabetes mellitus and aging. Several secondary products of lipid oxidation, especially malondialdehyde and 4-hydroxynonenal, have been shown to react with biological components such as proteins, amino acids and DNA. Malondialdehyde is formed both enzymatically and non-enzymatically, has been implicated in aging, mutagenesis and carcinogenesis (Ramarathnam *et al.*, 1995).

The existing literature, support the role of advanced glycoxidation end products (AGE) in the pathogenesis of diabetic complications; micro- and macro- angiopathy which underlie delayed diabetic wound healing types (Peppia & Raptis, 2011). In addition, a large body of evidence support a direct negative effect of AGE in the wound healing process by their interference with various components involved in the cascade following skin injury (Peppia & Raptis, 2011). Endogenously formed or exogenously derived oxidation products, in a similar manner, affect negatively the wound healing mechanism in diabetes. It is clear that further studies are needed to clarify the exact role of oxidation products in the impaired diabetic wound healing and offer possible new therapeutic strategies (Peppia & Raptis, 2011).

Oxidative stress in diabetic wound healing rats was measured by the resulting oxidative products such as LHP and AOPP, related to cytotoxicity and delayed wound healing, both of which are stable markers of oxidative stress.

2.5.2 Protein Oxidation Products

An imbalance between antioxidant and oxidant-generating systems were resulting in oxidative stress. As plasma proteins are the critical targets for oxidants, AOPP is a novel marker of oxidative stress, detected in plasma or purified HSA. Measurement of AOPP is a reliable marker to estimate the degree of oxidant-mediated protein damage and to predict the potential efficacy of therapeutic strategies aimed at reducing such an oxidative stress (Witko-Sarsat *et al.*, 1996).

Advanced oxidation protein product was first described by Witko-Sarsat *et al.*, (1996). Advanced oxidation protein product OPP are formed during oxidation stress by the action of chlorinated oxidants, mainly chloramines and hypochlorous acid. They are structurally similar to advanced glycation end-product (AGE, induction of pro-inflammatory cytokines and adhesive molecules).

Proteins are major targets for radicals and other oxidants, and the use of radical-mediated protein oxidation products as sensitive markers for oxidative damage in mammalian system has been studied and reviewed extensively (Davies *et al.*, 1987). Protein oxidation products and carbonyl derivatives of proteins may result from oxidative modifications of amino side chains, reactive oxygen-mediated peptide cleavage, and reactions with lipid and carbohydrate oxidation products. Studies have shown that the presence of carbonyl groups in proteins may indicate that the proteins have been subjected to oxidation (Ergul *et al.*, 2004). Advanced oxidation protein product is used as biomarker for protein oxidation.

The hyperglycaemia associated with diabetes can cause tissue damage in two ways, the first pathway is the intracellular hyperglycaemia which can adversely affect cellular functions and second pathway is the non-enzymatic glycation of proteins (glycooxidation). Advanced oxidation protein products and AGE are important biomarkers associated and

correlated with oxidative stress, metabolic disorder and atherosclerotic risk markers in elderly diabetic subjects (Gradinaru *et al.*, 2013). Advanced oxidation protein product and AGE markers could be valuable in predicting vascular complication in Type-2 diabetes and the onset of diabetes (Gradinaru *et al.*, 2013). Advanced oxidation protein product and AGE form on intra- and extracellular proteins, lipids, nucleic acids with complex structures that generate cross-linking, which could alter intracellular signalling gene expression, released of pro-inflammatory molecules and free radicals that contribute towards the pathology of diabetic complication. Increased intracellular ROS cause defective angiogenesis and cause long-lasting epigenetic changes. In addition, hyperglycemia and a number of hyperglycemia-related factors have been linked to impaired diabetic wound healing including AGE and AOPP. The mechanism of action could be directly by their interference with a variety of components involved or indirectly through their association with diabetic neuropathy and/or angiopathy (Peppia *et al.*, 2009).

2.5.3 Lipid Peroxidation

Lipids, in particular polyunsaturated fatty acids and their esters (PUFA) are quite susceptible to oxidation and their oxidation products may serve as appropriate biomarkers. The excess production of ROS, particularly hydroxyl radicals, can easily initiate lipid oxidation in the cell membrane, resulting in the formation of lipid peroxides and lipid hydroperoxides. Lipid hydroperoxides are primary lipid peroxidation products formed when omega-6 polyunsaturated fatty acids, such as linoleic acid, react with free radical species (Esterbauer, 1996). Takayama *et al.*, (1992) reported that lipid hydroperoxides are produced as result of ischaemic and reperfusion in tissues, including the heart and muscle. The reactivity of lipid hydroperoxides is such that they are damaging to proteins within tissues and the ability of these species when applied exogenously to participate in cardiac

dysfunction (Thollon *et al.*, 1995). Since lipid hydroperoxides are produced in the heart during oxidative stress and it is able to elicit cardiac dysfunction, it is possible that a component of the injury which accompanies ischaemia and reperfusion might be a result of lipid hydroperoxide mediated cardiac protein modification. A growing number of studies have shown a role for reactive lipid oxidation products, such as lipid hydroperoxides and its breakdown product hydroxynonenal, in the initiation of redox-sensitive signal transduction pathways (Herrlich & Bohmer, 2000).

Lipid peroxidation is an example of oxidative damage in lipoproteins, cell membranes, and other lipid-containing structures. Peroxidative modification of unsaturated phospholipids, cholesterol, and glycolipids can occur in reactions triggered by free radical species and non-radical species. Lipid hydroperoxides (LHPs) are prominent non-radical intermediates of lipid peroxidation whose identification can often provide valuable mechanistic information (Wijeratne & Cuppett, 2006). An experiment using oleic acid hydroperoxides induced oxidative stress damage showed that as the concentration of lipid hydroperoxides increases, cell membrane damage and DNA damage were significantly ($p < 0.05$) increased, indicated by conjugated diene concentration (Wijeratne & Cuppett, 2006). Since the reactivity of lipid hydroperoxides is such that they are damaging to proteins within tissues and cell, which may affect wound healing, and measuring the *in vivo* lipid hydroperoxides in diabetic wound healing animal could indicate the oxidative status of the experimental animal.

3. MATERIALS AND METHODS

3.1 Preparation of Aqueous Extract of *G. lucidum*

The fresh fruiting bodies of *G. lucidum* were obtained from Ganofarm Sendirian Berhad, a mushroom farm in Tanjung Sepat, Selangor, Malaysia. The production of *G. lucidum* was reported previously (Chin *et al.*, 2008; Chin *et al.*, 2009). One kilogram of air-dried fruiting bodies of *G. lucidum* was subjected to hot water extraction (1:10 (w/v) at 100°C for 8 h. The resulting aqueous extract was filtered, freeze-dried at -50°C by and stored at -20°C prior to use. Analysis of marker components (total polysaccharides, ganoderic acid A and adenosine), microbial count (AOAC, 1990) and heavy metal content (APHA, 1984) were performed in Nova Laboratory, Sepang, Malaysia using proprietary methodology (Appendix A, i, pp. 129).

3.2 Preparation of Mushroom Treatment Cream

Aqueous cream was obtained from the Department of Pharmacy, Faculty of Medicine, University of Malaya (a product of Sunward Pharmaceutical Sendirian Berhad, MAL 19920890 X). The freeze-dried aqueous extract was mixed homogeneously with aqueous cream at concentrations of 5%, 10%, 15% and 20% (w/w).

3.3 Preparation of Experimental Animals

Healthy adult male *Sprague Dawley* (SD) rats were obtained from the animal house, Faculty of Medicine, University of Malaya. The rats were divided randomly into twelve groups of six rats each. Each rat weighed between 180 to 250 g (about 6 to 8 weeks old)

and was housed separately (one rat per cage). The animals were fed on standard pellet diet and tap water. The research conformed to the Principles of Laboratory Animal Care and was approved by the Ethics Committee of University of Malaya with the Ethic No. ISB/14/10/2009/CPG (R). All animals received 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 Institutes of Health.

3.4 Animal Grouping

The animals were divided into two main groups; consisting of 36 normal rats (group 1) in 6 subgroups of 1a – 1f, and 36 streptozotocin-induced diabetic rats (group 2) in 6 subgroups of 2a – 2f. The model mouse in group 2 is for Type-1 diabetes wound study. Group 1a and group 2a rats were treated with aqueous cream as negative control; group 1b and group 2b rats were treated with commercial Intrasite gel as positive control while rats in groups 1c - 1f; and groups 2c - 2f were treated with mushroom extract incorporated treatment creams as in Table 3.1.

Table 3.1: Experimental animals grouping and treatments

Normal rats grouping	Diabetic rats grouping	Treatment
1a	2a	Aqueous cream
1b	2b	Intrasite gel
1c	2c	Aqueous cream containing 5% mushroom extract
1d	2d	Aqueous cream containing 5% mushroom extract
1e	2e	Aqueous cream containing 5% mushroom extract
1f	2f	Aqueous cream containing 5% mushroom extract

3.5 Diabetes Induction

Streptozotocin (STZ) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Diabetes mellitus was induced in six groups of rats (2a – 2f) by a single intraperitoneal injection of STZ at 45 mg/kg. Streptozotocin was previously dissolved in citrate buffer of 0.1 M with pH 4.0. After three days of STZ injection, rats were fast overnight before blood was drawn from the tail for blood glucose reading. Glucose level was estimated using a glucometer (Ames, Bayer Diagnostic). Rats with fasting blood glucose levels higher than 10 mmol/L (or 200 mg/dL) were considered diabetic and were included for the experiment (Morton & Malone, 1972). This study was initiated to develop an animal model of Type-1 diabetes.

3.6 Excision Wound Creation

Excision wounds were created on the experimental animals after an overnight fast. Blood glucose readings were taken prior to wound creation. The animals were anaesthetised with about 2 mL of diethyl ether (Sigma, 98% purity). The skin was shaved using an electric clipper at the dorsal neck region. It was then disinfected with 70% (v/v) alcohol, and 0.5 mL of lignocaine HCl (2%, 20 mg/ mL) was injected as a local anaesthetic agent. The area of wound was outlined with methylene blue using a circular stencil. An overall thickness of the excision wound of 2.0 cm in diameter was created as described by Nayak & Pereira (2006). Care was taken to avoid injury to the muscle layer, and the tension of skin was kept constant during the procedure. The wound creation was depicted in Plate 3.1.



Plate 3.1: Full thickness skin excision.

3.7 Topical application of Treatment Cream

The wounds of experimental animals were treated according to the treatment group as described in Table 3.1. Immediately after the wound creation, the whole wound area was covered with treatment cream and the rats were placed individually in their cages. Normal and diabetic groups of rats were labelled and housed in two different rows of rack. Applications of cream were performed twice a day without damaging the wounds. The wound was cleaned once a day using commercial antiseptic solution to prevent from microbial infection.

3.8 Determination of the Period of Re-Epithelialisation

The wounds of all animals under the different treatments were observed daily. The period of re-epithelialisation was assessed by counting the number of days required for complete healing without any residual raw wound (Ponrasu & Suguna, 2012).

3.9 Estimation of the Wound Closure

The wound area was measured to evaluate the wound closure. The wound area (mm^2)

was measured at day 0, 4, 8, 12 and 16 post operation using transparency paper and a permanent marker. Total wound area of the rats was estimated on the transparency with grids (5x5) mm² (Plate 3.2).

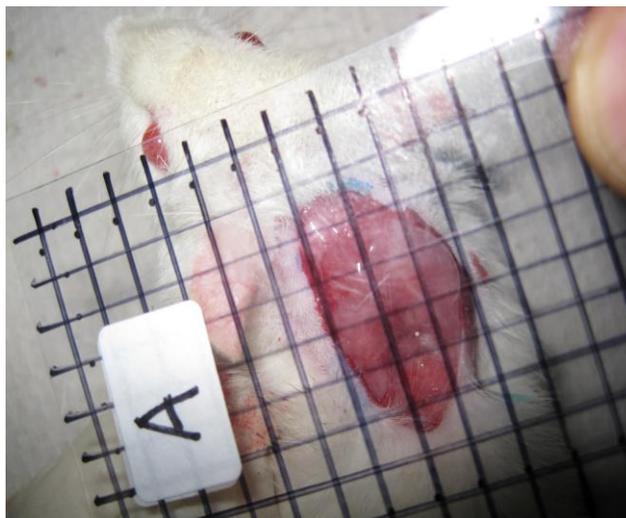


Plate 3.2: Transparency sheet with grid (5x5) mm² used for estimation of wound area (Nayak & Pereira, 2006).

To determine the rate of wound closure, the change in wound size was calculated as the percentage of wound area that had healed. Percentage of wound closure was calculated using the following formula (Nayak & Pereira, 2006):

$$\text{Percentage of wound closure (\%)} = (\text{Wound area on day 0} - \text{Wound area on post-operation day}) / (\text{Wound area on day 0}) \times 100\%$$

3.10 Histological Evaluation of Healed Wound

The skin biopsies taken from healed wounds and the surrounding tissues were fixed in 10% (v/v) buffered formalin and processed with the automated tissue processing machine (Appendix A, x, pp. 137). Three sections (5 μm thickness) of healed wound tissue of the rats were prepared for hematoxylin and eosin (H & E) for cellular and Masson's

Trichrome (MT) staining for collagen (Carson & Feida, 1990). Staining techniques of H & E is given in Appendix A , xi, pp 138 and MT is given in Appendix A, xii, pp 139. Sections of each wound were examined by light microscopy. Scar width (mm) which is the junction gap between the normal dermis and dermis in the wound tissues was measured (Al-Bayaty & Abdulla, 2012). The morphological changes (fibroblast, inflammatory cell, neovascularisation, and collagen) were evaluated.

3.11 Determination of *in Vivo* Antioxidant Capacity of Experimental Rats

Antioxidant activity in the blood serum was determined by *In vivo* cupric ion reducing antioxidant capacity (CUPRAC) method (Apak *et al.*, 2004), which utilises copper (II)-neocuproine reagent as the chromogenic oxidising agent. The mixture of one mL of CuCl_2 (10^{-2} M), neocuproine (7.5×10^{-3} M) and ammonium acetate buffer solution (1 M) was added into a cuvette. Then, 1090 μL of distilled water with 10 μL of blood serum was added into the reagent mixture and incubated at room temperature ($25 \pm 2^\circ\text{C}$) for 30 minutes. The absorbance at 450 nm was recorded against a reagent blank. The result of antioxidant activity was expressed as absorbance at 450 nm against blank. Ascorbic acid was used as positive control. The sample of serum was analysed in triplicates for absorbance reading, for each group ($n = 6$) of experimental animals.

3.12 Assessment of Oxidative Damage

3.12.1 Advanced Oxidation Protein Products (AOPPs) Assay

The hyperglycaemia associated with diabetes can cause tissue damage in two ways, the first pathway is the intracellular hyperglycaemia which can adversely affect cellular functions and second pathway is the non-enzymatic glycation of proteins (glycooxidation).

Advanced Oxidation Protein Products and advanced glycation end product (AGE) are important biomarkers associated and correlated with oxidative stress, metabolic disorder and atherosclerotic risk markers in elderly diabetic subjects (Gradinaru *et al.*, 2013). Advanced Oxidation Protein Products and AGE markers could be valuable in predicting vascular complication in Type-2 diabetes and the onset of diabetes (Gradinaru *et al.*, 2013). Advanced Oxidation Protein Product and AGE form on intra and extracellular proteins, lipids, nucleic acids with complex structures that generate cross-linking, which could alter intracellular signalling gene expression, released of pro-inflammatory molecules and free radicals that contribute towards the pathology of diabetic complication and wound healing.

Advanced Oxidation Protein Product was determined by the method of Witko-Sarsat *et al.*, (1996). The premixed reagent mixture was prepared by adding 81 mL of phosphate buffer saline solution, 15 mL of 50% acetic acid (v/v) and 4 mL of potassium iodide. Then, 18 μ L of serum sample was added to 200 μ L of reagent mixture in 96-well micro-titer plate. A series of standards were prepared by adding the premixed reagent to 18 μ L of chloramines-T solution (0-500200 μ mol/L). The absorbance of reaction mixture was immediately read at 340 nm and was recorded against a reagent blank. Advanced oxidation protein products were expressed as μ mol/L chloramine-T equivalents. Each sample of plasma was triplicated for absorbance reading, n = 6 for each group of experimental animal.

3.12.2 Lipid Hydroperoxide (LHP) Assay

It has been demonstrated that free radicals, ROS, and oxidative products, such as LHP, engage in tissue injuries and in the onset and progression of degenerative diseases in human. Lipid peroxidation is a well-known example of oxidative damage in lipoproteins, cell membranes, and other lipid-containing structures. Peroxidative modification of

glycolipids, unsaturated phospholipids, and cholesterol can take place in reactions triggered by free radical species and non-radical species. Lipid hydroperoxides (LOOHs) are prominent non-radical intermediates of lipid peroxidation whose identification can often provide valuable mechanistic information. A derangement in redox homeostasis, due to sustained levels of oxidative stress and related mediators, can play a significant role in the pathogenesis of major human diseases characterized by chronic activation of wound healing, chronic inflammation, and tissue fibrogenesis (Wijeratne & Cuppett, 2006).

Lipid hydroperoxide value was measured according to Esterbauer and Cheeseman, (1990). Malondialdehyde (MDA) was assayed as a marker of lipid peroxidation using colorimetric reaction, which uses 1-methyl-2-phenylindole (MPI) as chromogen. A total of 150 μL of serum sample was added to 375 μL of MPI in acetonitrile and 225 μL HCl (5M) in microcentrifuge tube and incubated in a water bath at 45°C for 40 minutes. Tetraethoxypropane (TEP) was used as standard solution at a concentration of 0, 2.5, 5, 10, 15 and 20 μM . After centrifugation at 10,000 g for 5 minutes, reaction mixture was read at absorbance 586 nm in an ELISA reader (UV 1601 spectrophotometer, Shimadzu, Japan). Concentration of lipid hydroperoxide was expressed as $\mu\text{mol/L}$ of TEP equivalents. Each sample of serum was, $n=6$ for each group of experimental animal.

3.13 Statistical Analysis

The results were expressed as mean \pm standard error mean (SEM), analysed by *STATISTICA Version 8 Automated Neural Networks*. Differences between groups were tested by one way analysis of variance (ANOVA) followed by Duncan's multiple comparison trials. The p values less than 0.05 were considered statistically significant and $n=6$. All antioxidant and oxidation assays were performed in triplicates.

4. RESULTS

4.1 Yield and Analysis of Aqueous Extract of *G. lucidum*

The extraction yield of freeze-dried aqueous extract from 1kg of dried fruiting bodies of *G. lucidum* was 8.98g. The appearance of fruiting bodies of *G. lucidum* from Ganofarm Sendirian Berhad is depicted in Plate 4.1(a) and the freeze-dried aqueous extract powder was brown in colour as depicted in Plate 4.1(b).

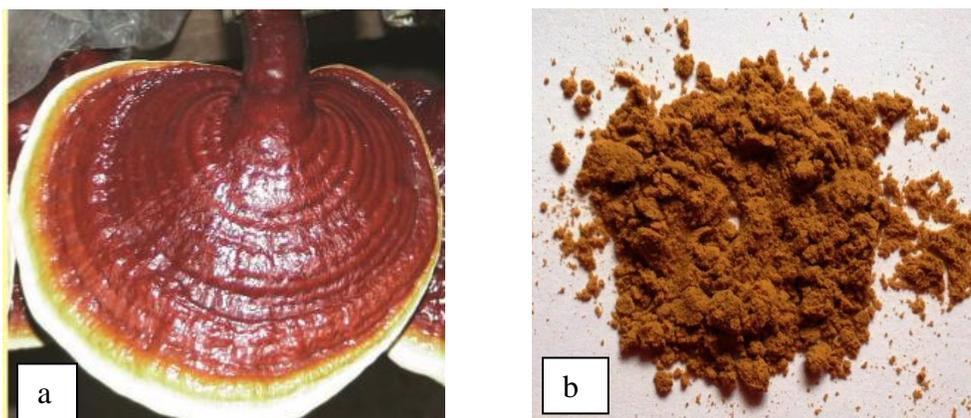


Plate 4.1: Fresh fruiting bodies and the freeze-dried aqueous extract powder of *G. lucidum*.

In the analysis of selected marker compounds, total polysaccharide measured by UV was 25.1% (w/w), while ganoderic acid A and adenosine quantified by HPLC were 0.45% (w/w) and 0.07% (w/w) respectively (Table 4.1). The amount of heavy metals in the extract measured by atomic absorption spectrophotometer, indicated as < 5.0 ppm of arsenic, < 10.0 ppm of lead, < 0.5 ppm of mercury and < 0.3 ppm of cadmium (Table 4.1) which is in the food safety area. Microbial tests revealed that total bacterial count, yeast and mould count, as well as enterobacteriaceae was not more than 10^5 , 10^4 , and 10^5 cfu/g. *Salmonella*

spp., *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were not detected in the aqueous extract of *G. lucidum* (Table 4.2).

Table 4.1: Components and heavy metal tests of aqueous extract of *G. lucidum*

Marker Components	Contents (%)
Total polysaccharides	25.10±1.26
Ganoderic acid A	0.45±0.02
Adenosine	0.07±0.00
Heavy Metal Tests	Contents
Arsenic	Not more than 5.0 ppm
Lead	Not more than 10.0 ppm
Mercury	Not more than 0.5 ppm
Cadmium	Not more than 0.3 ppm

Table 4.2: Microbial Load Analysis

Microbial	Total count
Total bacteria count	Not more than 10 ⁵ cfu/g
Yeast and mould count	Not more than 10 ⁴ cfu/g
Enterobacteriaceae	Not more than 10 ³ cfu/g
<i>Salmonella</i> spp.	Absent
<i>E. coli</i>	Absent
<i>S. aureus</i>	Absent
<i>P. aeruginosa</i>	Absent

4.2 Diabetes Induction

After the injection of STZ, an increase in blood glucose levels was recorded over time, and this was accompanied by a reduction in body weight compared to their body weight at day 0. STZ-treated rats lost 11.33 ± 5.89 g and 20.83 ± 8.66 g body weight at 7 days and 14 days post-STZ injection, respectively, while the normal controls gained 32.25 ± 16.28 g and 58.00 ± 19.45 g at 7 and 14 days, respectively.

Blood glucose levels in diabetic rats used in the present study were consistently higher than 200 mg/dL and these levels did not drop but increased during the period of treatment. The blood glucose reading on 7 days post-injection was 19.23 ± 7.94 mmol/L in negative control group, 20.67 ± 3.79 mmol/L in positive control group, 21.78 ± 3.04 mmol/L in 5% extract treated group, 20.65 ± 6.26 mmol/L in 10% extract treated group, 23.50 ± 3.15 mmol/L in 15% extract treated group and 23.78 ± 5.37 mmol/L in 20% extract treated group. Six (15%) out of forty STZ-induced diabetic rats died during the first week, four rats died due to hyperglycemia (> 33 mmol/L) or 'diabetic coma' and two rats died of hypoglycemia (fasting blood glucose < 3 mmol/L). Some rats were weak after loss of body weight due to untreated hyperglycemia at the end of sixteen days experiment.

4.3 The Effects of Aqueous Extract of *G. lucidum* on Wound Healing

4.3.1 Effect of *G. lucidum* Extracts on Wound Re-epithelialisation

Period of re-epithelialisation is the total number of days taken for complete healing of the wounds after operation. The re-epithelialisation periods for normal and diabetic rats are shown in Fig 4.1.

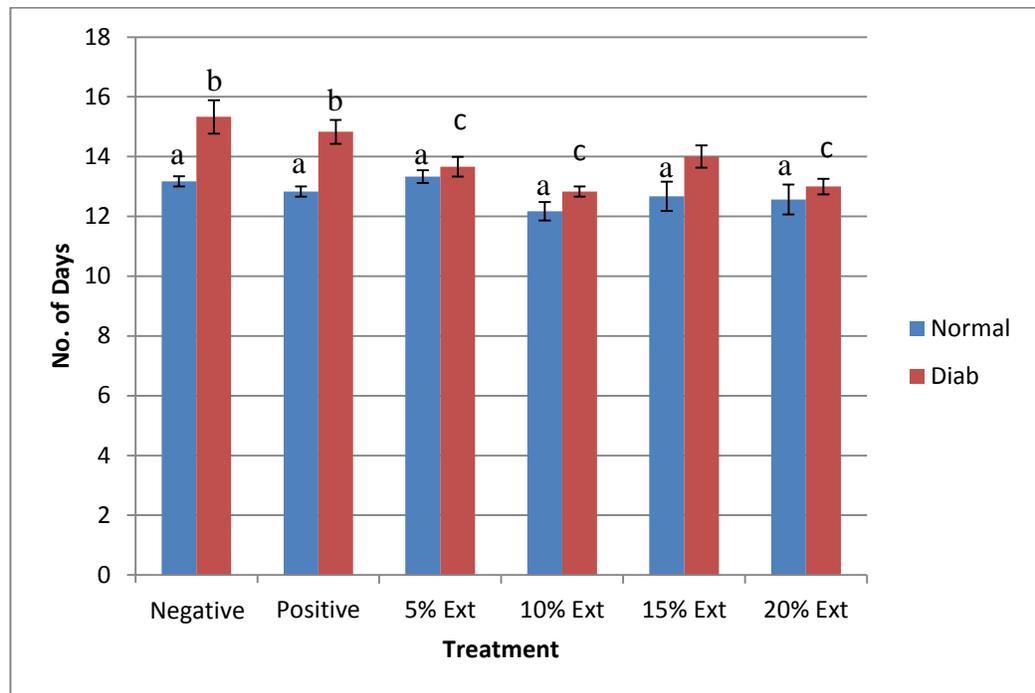


Figure 4.1: The effects of the extracts of *G. lucidum* on wound re-epithelialisation for normal and diabetic rats. Data are expressed as means \pm standard error, n=6. Means with (*) were significantly different ($p < 0.05$) by ANOVA; Duncan's multiple comparison test. Abbreviation: Ext: Aqueous cream containing 5%, 10%, 15% and 20% *G. lucidum* extract, Diab: Diabetes.

In normal rats (Group 1), the wounds took 12 to 14 days for complete healing. Wounds of normal rats treated with aqueous cream (negative control) took 13.17 ± 0.17 days, whereas wound treated with Intrasite gel (positive control) took 12.83 ± 0.17 days to heal completely. Although the wounds of normal rats (Group 1) treated with mushroom extract there was no significant difference ($p < 0.05$) in the re-epithelialisation period, the wounds treated with 10%, 15% and 20% (w/w) extract of *G. lucidum*, the healing time was shorter and in ascending order when compared to control, with 12.17 ± 0.31 , 12.67 ± 0.49 and 12.57 ± 0.50 days respectively (Fig 4.1), which is comparable to the time taken for healing with Intrasite gel (positive control). Furthermore there was no significant reduction in re-epithelialisation period in wounds treated with increasing concentration of aqueous extract of *G. lucidum*. This indicates that normal rats have the inner immunity to heal

naturally and healed faster with the additional effect of mushroom extract. The physiological cellular response to tissue injury in normal rats was normal and no disturbances in the basic cell function. Injury of healthy normal rats normally results in a nearly complete recovery of the anatomic and functional integrity in the wounded area.

In contrast, the healing time of wounds in diabetic rats (Group 2) was delayed compared to wounds of normal rats (Group 1) (Fig. 4.1). Wounds of diabetic rats treated with aqueous cream alone (negative control) took 15.33 ± 0.56 days while wounds treated with Intrasite gel (positive control) took 14.83 ± 0.40 days for re-epithelialisation. Healing time of wounds in positive control diabetic rats was not significantly different ($p < 0.05$) when compared to negative control rats. The wounds of diabetic rats treated with aqueous cream containing 5%, 10%, 15% and 20% (w/w) mushroom extract healed in 13.66 ± 0.33 , 12.83 ± 0.12 , 14.00 ± 0.37 and 13.00 ± 0.26 days respectively, which is significantly ($p < 0.05$) faster than negative and positive control diabetic rats. Notably, wounds of diabetic rats treated with aqueous cream containing 10% mushroom extract healed in the shortest period among the groups. However, the re-epithelialisation period of wounds in diabetic rats treated with aqueous cream containing 15% and 20% (w/w) aqueous extract were not faster compared to the wounds treated with 10% (w/w) mushroom extract. Therefore the use of 10% mushroom extract is sufficient to enhanced wound healing. Due to the systemic character of diabetes, disturbance in several basic cell functions which appear to contribute to the impaired wound healing in diabetic rats. Pathogenesis of wound healing in diabetes mainly cause by dysfunctional wound cells and by imbalances in key proteases, cytokines and growth factors.

4.3.2 Effect of *G. lucidum* Extracts on the Rate of Wound Closure

To determine the rate of wound closure, the change in wound size was calculated as the percentage of wound area that had healed. Wound closure area was measured and the percentage of the healed wound area was calculated on day 4, 8 and 12 post-operation. Results of wound closure in normal rats are shown in Fig. 4.2.

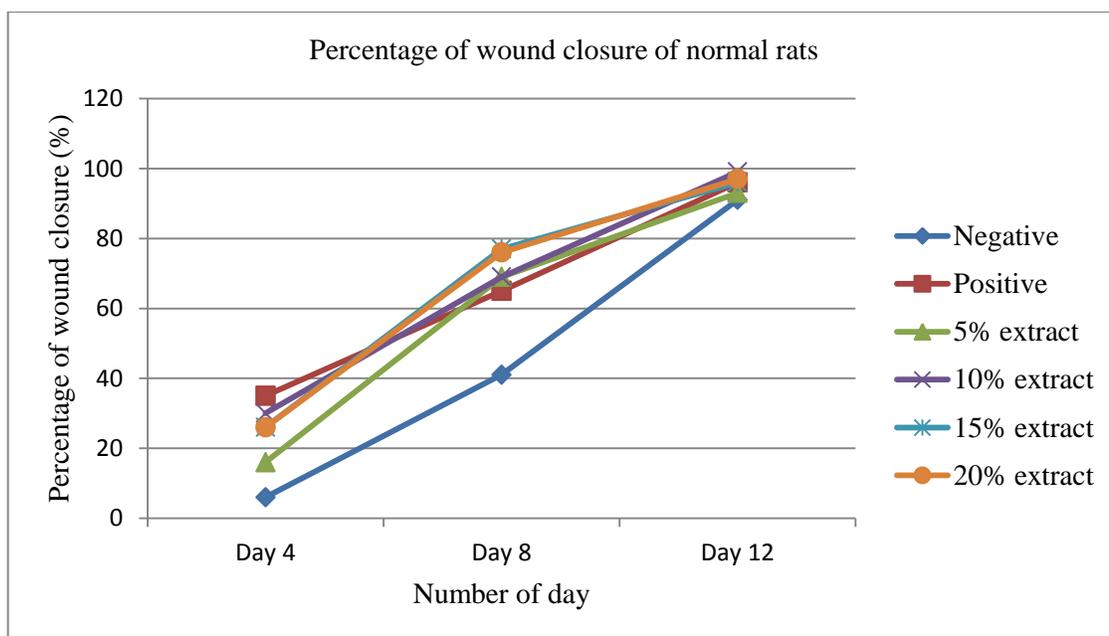


Figure 4.2: The effects of the extracts of *G. lucidum* on wound closure rate in normal rats. Data are expressed as means \pm standard error, n=6. Extract: Aqueous cream containing 5%, 10%, 15% and 20% *G. lucidum* extract.

The wound closure of normal rats at day 4 post-operation treated with aqueous cream containing 10%, 15% and 20% mushroom extract was 75.29 ± 14.02 mm² (30%), 64.21 ± 16.75 mm² (25%) and 66.20 ± 14.91 mm² (26%) respectively, which was not significant different compared to the wound treated with commercial Intrasite gel with wound closure 88.40 ± 13.10 mm² (35%). Wound treated with aqueous cream (negative control group) and aqueous cream containing 5% (w/w) mushroom extract were closed 15.57 ± 3.28 mm² (6%) and 40.55 ± 6.52 mm² (16%) respectively. All wounds in normal rats

showed contraction, but no wounds from any of the treatments had completely healed by day 4 post-operation. This indicated that the aqueous cream containing mushroom extract enhanced wound healing in early inflammatory stage.

Wound closure of normal rats at day 8 post-operation (Fig. 4.2) was led by group treated with aqueous cream containing 15% followed by 20%, 10%, 5%, Intracel gel and aqueous cream, with wound closure area of 192.38 ± 23.36 (77%), 189.57 ± 32.78 (76%), 172.46 ± 16.20 mm² (69%), 172.11 ± 19.05 mm² (69%), 163.45 ± 19.09 mm² (65%) and 102.54 ± 20.16 mm² (41%) respectively. It seems that wounds of rats treated with Intracel gel which contracted the fastest at day 4 post-operation has slowed down in the rate of wound closure on day 8 post-operation. This indicated that aqueous cream containing mushroom extract has better effect on wound closure at inflammatory and proliferation stages of wound healing. No wounds were completely healed at day 8 post-operation.

All the wounds in normal rats were near to complete healing at day 12 post-operation, led by aqueous cream containing 10%, followed by 20% and 15% mushroom extract, Intracel gel (positive control), 5% mushroom extract and aqueous cream (negative control) with wound closure area of 247.71 ± 2.55 mm² (99%), 241.92 ± 6.79 mm² (97%), 240.59 ± 9.24 mm² (96%), 240.28 ± 10.28 mm² (96%), 233.16 ± 18.73 mm² (93%), and 226.72 ± 12.38 mm² (91%) wound closure. The results indicated that wounds treated with aqueous cream containing 10%, 15% and 20% mushroom extract healed significantly ($p < 0.05$) faster than the wounds treated with aqueous cream (negative control). No significant difference in wound closure was found between 10%, 15% and 20% of mushroom extract by student-*t* test. The use of 10% mushroom extract incorporated cream is sufficient to accelerate wound healing in normal rats and the effect of wound healing was comparable to commercial Intracel gel. Overall, the results showed a steady increase of

wound closure rate from day 4 to day 12 post-operation in rats wound treated with Intrasite gel (positive control) and wound treated with cream containing 10% mushroom extract. Aqueous cream containing 10% mushroom extract is as good as commercial Intrasite gel in healing of wound in healthy normal rats. The rate of wound closure in diabetic rats is shown in Fig. 4.3.

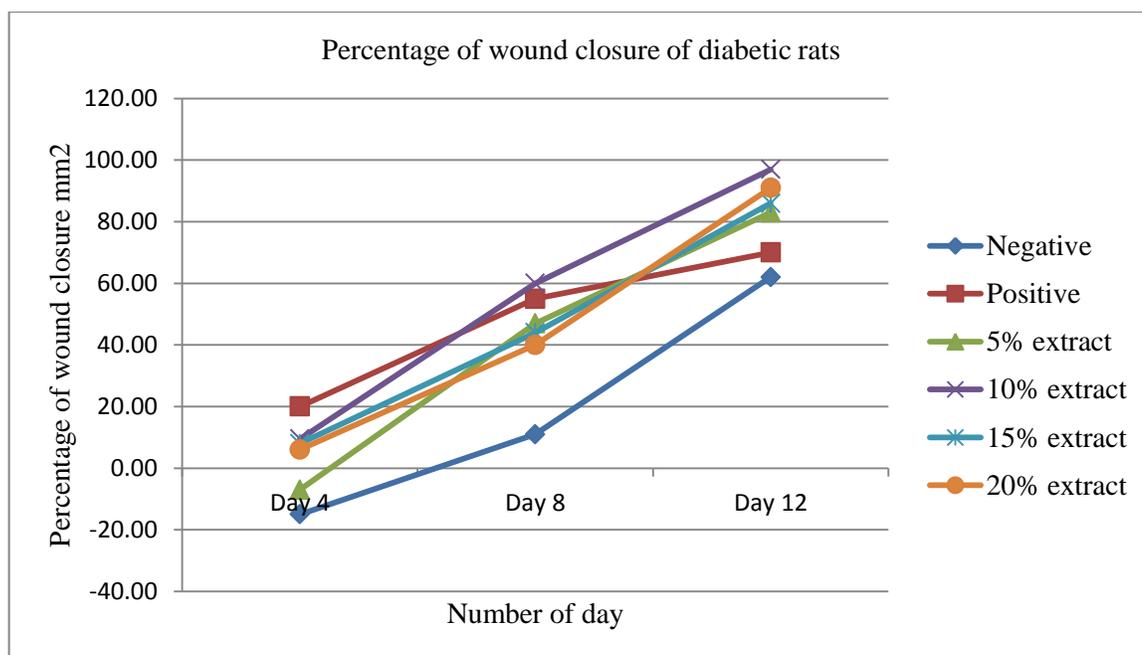


Figure 4.3: The effects of the extracts of *G. lucidum* on wound closure rate in diabetic rats. Data are expressed as means \pm standard error, n=6. Extract: Aqueous cream containing 5%, 10%, 15% and 20% of *G. lucidum* extract.

Results demonstrate that the wound closure of diabetic rats (Group 2) at day 4 post-operation treated with aqueous cream containing 5%, 10%, 15% and 20% mushroom extract was $-17.81 \pm 2.52 \text{ mm}^2$ (-7%), $23.79 \pm 2.71 \text{ mm}^2$ (9.5%), $20.78 \pm 4.62 \text{ mm}^2$ (8%) and $14.44 \pm 4.43 \text{ mm}^2$ (6%) respectively, while treatment with aqueous cream and Intrasite gel showed $-126.66 \pm 15.94 \text{ mm}^2$ (-51%) and $49.14 \pm 14.18 \text{ mm}^2$ (20%) wound closure respectively (Fig. 4.3). Notably, delayed in wound healing was observed in wounds of

diabetic rats (Group 2) compared to normal rats (Group 1). Among the diabetic groups Intarsite gel treated rats showed the greatest ($p < 0.05$) wound closure. Wounds treated with Intraspine gel and aqueous cream containing 10%, 15% and 20% mushroom extract showed contraction whereas wounds treated with aqueous cream containing 5% mushroom extract and aqueous cream (negative control) showed expansion (-ve value in wound closure). Intraspine treated wound showed sign of healing and the wound closure was significantly fastest. This indicated that Intraspine gel stimulate the inflammatory, fibroblast proliferation and keratinocytes formation, thus a more rapid maturation of granulation tissue (Moyer *et al.*, 2002). In contrast, the delayed wound healing in diabetic rats treated with aqueous cream (negative control) and 5% mushroom extract is believed resulting from the prolonged inflammatory reaction, which leads to increase levels of proteases that destroy components of ECM and damage to growth factors and their receptors that are essential for healing process (Schultz *et al.*, 2005).

From the result (Fig. 4.3), wound closure in diabetic rats on day 8 was led by group treated with aqueous cream containing 10% mushroom extract, followed by Intraspine gel, aqueous cream containing 5%, 15%, 20%, mushroom extract and lastly aqueous cream (negative control), with wound closure area of $149.12 \pm 16.50 \text{ mm}^2$ (60%), $136.86 \pm 22.85 \text{ mm}^2$ (55%), $120.98 \pm 6.13 \text{ mm}^2$ (47%), $110.76 \pm 16.09 \text{ mm}^2$ (44%), $101.04 \pm 23.19 \text{ mm}^2$ (40%) and $39.23 \pm 6.13 \text{ mm}^2$ (11%) respectively. It seems that wounds of rats treated with Intraspine gel contracted the fastest at day 4 and gradually slowed down in the rate of wound closure when compared to wounds treated with aqueous cream containing 10% mushroom extract. No wounds were completely healed by day 8 post-operation. A rapid increase of wound contraction on day 8 post-operation was observed in all the diabetic groups compared to

day 4 post-operation. The healing process on day 8 post-operation may involve the inflammatory and proliferation phases, indicated that the ability of mushroom extract in enhancing cellular proliferation and collagen synthesis.

The wound closure at day 12 in diabetic rats wound treated with aqueous cream containing 5%, 10%, 15% and 20% mushroom extract was $217.12 \pm 30.11 \text{ mm}^2$ (83%), $242.08 \pm 7.81 \text{ mm}^2$ (97%), $213.90 \pm 16.13 \text{ mm}^2$ (86%), $226.62 \pm 13.51 \text{ mm}^2$ (91%), while wounds treated with aqueous cream (negative control) and Intrasite gel (positive control) was $155.81 \pm 20.65 \text{ mm}^2$ (62%), and $175.68 \pm 23.10 \text{ mm}^2$ (70%) respectively. The results indicated that wounds treated with aqueous cream containing 5%, 10%, 15% and 20% mushroom extract healed significantly ($p < 0.05$) faster than the wounds treated with aqueous cream (negative control). The improvement of wound contraction in diabetic rats wound treated with 10% mushroom extract was 35% better than those in negative control group. Overall, the results showed that rats wound treated with cream containing 10% and 15% mushroom extract have a steady increase in wound closure rate (straight line) as shown in normal rats with straight line graph. Notably wound healing effect of cream containing 10% mushroom extract is better than commercial Intrasite gel. At this stage, fibroblasts are stimulated to transform into myofibroblast that contract the wound matrix, collagen deposition (type I and type III) and vascular capillaries formation (Szabo *et al.*, 1995). Cream containing 10% mushroom extract enhanced wound healing in diabetic rats.

4.3.3 Macroscopic Analysis of Healed Wounds

Wound of the rats both normal and diabetic were photographed on day 4, 8, 12 and 16 for the macroscopic analysis. Morphology analysis of healed wound in normal and diabetic rats at 4, 8, 12, and 16 days post-operation were depicted in Plate 4.2 and Plate 4.3.

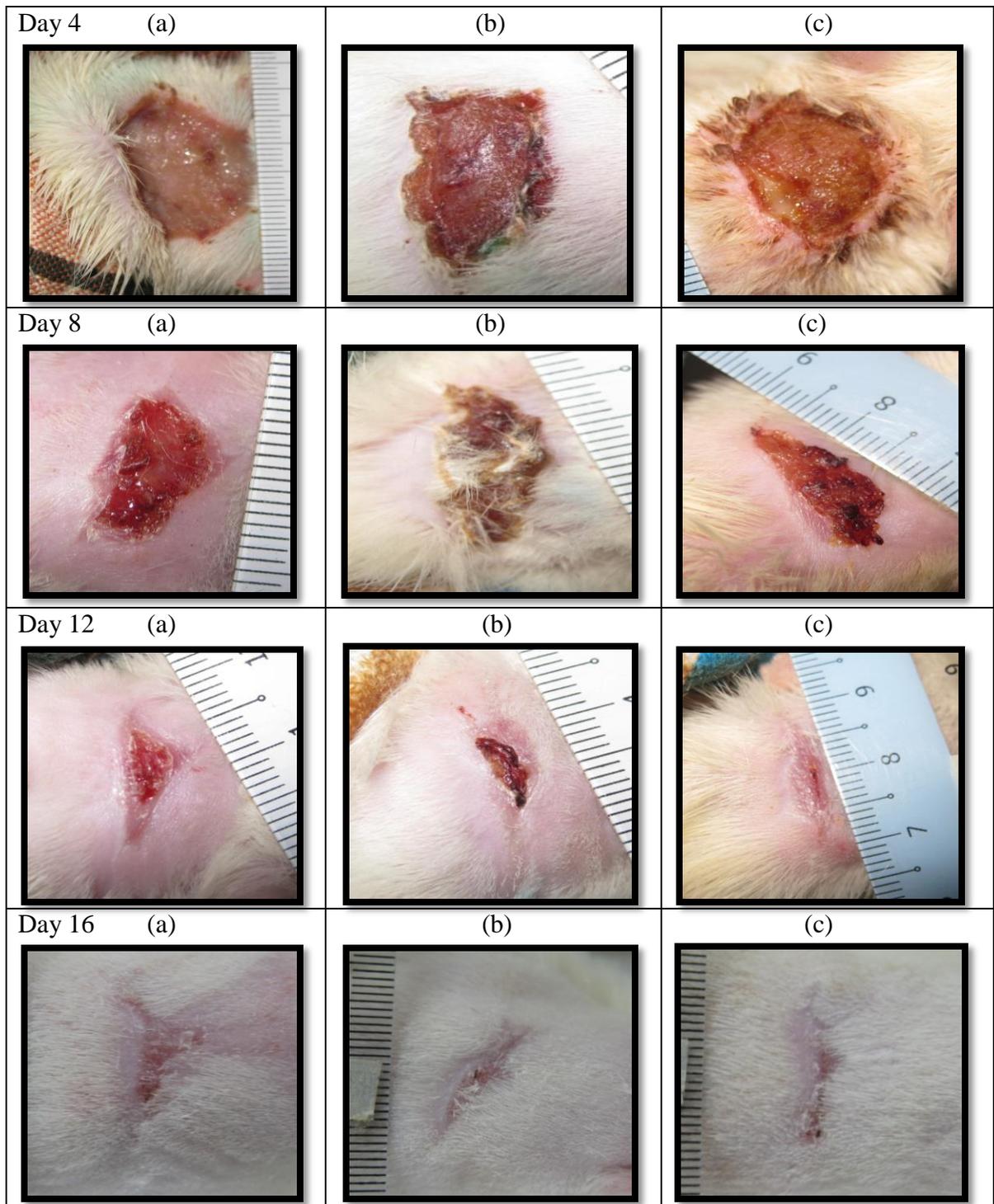


Plate 4.2: Macroscopic analysis of wound in normal rats (Group 1) at day 4, 8, 12 and 16 post-operation treated with aqueous cream (a), Intracite gel (b) and aqueous cream containing 10% mushroom extract (c).

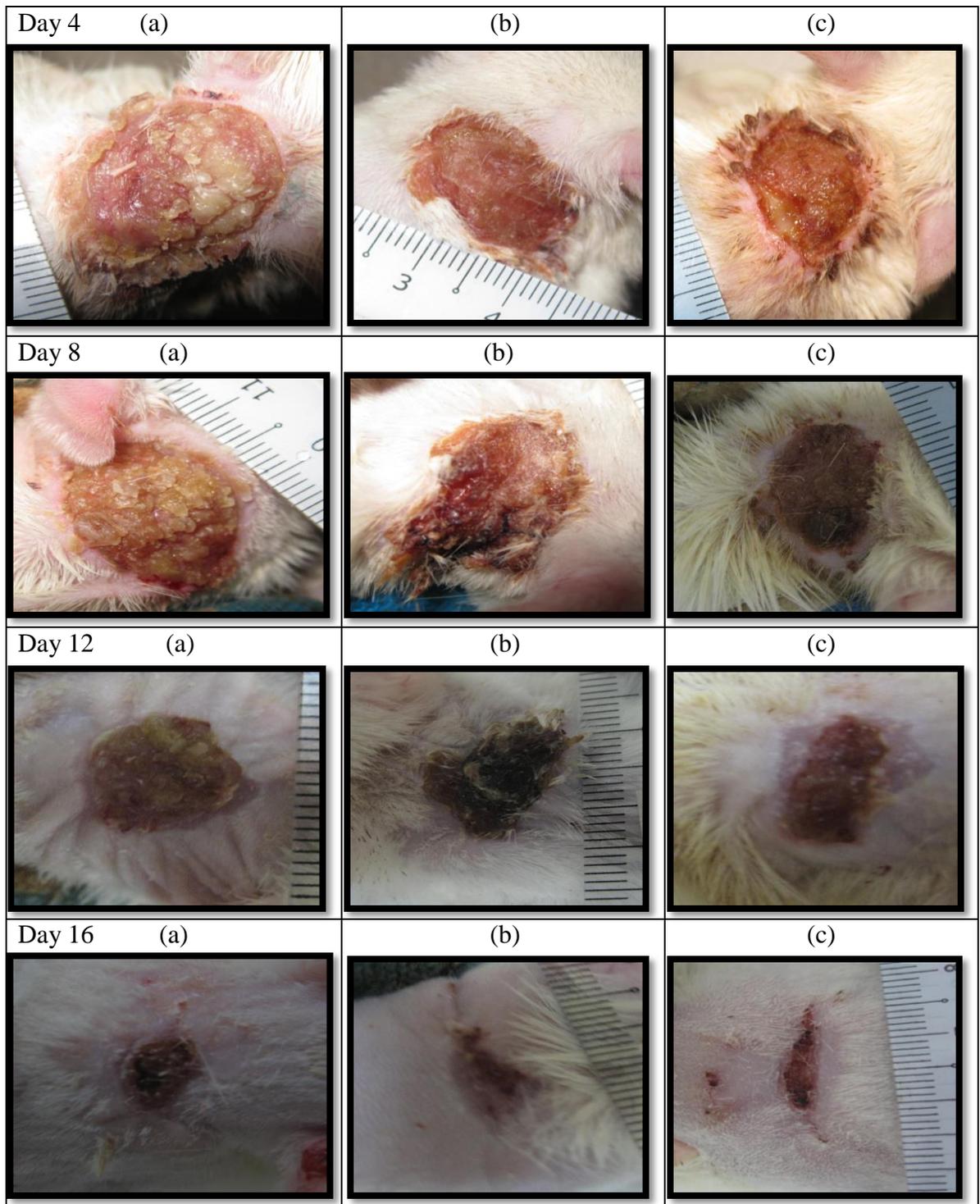


Plate 4.3: Macroscopic analysis of wound in Type-1 diabetic rats (Group 2) at day 4, 8, 12 and 16 post-operation treated with aqueous cream (a), Intracite gel (b) and aqueous cream containing 10% mushroom extract (c).

The result in Figure 4.2 showed that there was indication of healing in all wounds treated with aqueous cream, Intrasite gel and aqueous cream containing 10% of mushroom extract in normal rats. At day four post-operation, wounds surface in the normal rats in the group treated with Intrasite gel was dry while in the rats in groups treated with aqueous cream and aqueous cream containing 10% mushroom extract were moist. At day 8 post-operation wound surface of Intrasite had formed scaly and dry granulation tissue whereas wound surfaces treated with aqueous cream and aqueous cream containing mushroom extract had smooth and moist granulation tissues.

Wounds shrinkage was observed to nearly complete healing on day 12 post-operation. Scar tissue was observed at day 16 post-operation in all the normal rats, group of rats treated with aqueous cream containing 10% (w/w) mushroom extract the healing of the wound as a fine line was comparable to a sutured wound. The healing of normal rats was in a pattern trend even though the treatment cream given was different.

The wound macroscopic views at day 4, 8, 12 and 16 post-operation in diabetic rats are depicted in Plate 4.3. Morphology analysis of healed wound at day 4 post-operation showed that wound treated with aqueous cream containing 10% mushroom extract and Intrasite gel has granulation tissue formation with contraction of wound. The border of wounded skin of negative control rats was inflamed and exudates can be seen at the site of wounds (Plate 4.3), no sign of wound contraction was observed. This indicated that the proteolysis of granulation tissue occur at the injury skin of negative control rats treated with aqueous cream alone.

At day 8 and 12 post-operation, wounds treated with Intrasite gel showed the formation of hemorrhagic crust whereas wounds treated with aqueous cream containing mushroom extract showed moist granulation tissue on the surface, protected the wound

from infection. This indicated that larger wounds healing need a moist environment to preserve the granulation tissue and promote cellular migration. The natural tissue fluid is the physiological fluids that contain essential growth factors released by macrophages and fibroblasts. Lastly, the scar tissue of healed wound at day 16 post-operation was smaller in wound treated with aqueous cream containing mushroom extract compared to negative and positive control groups. Scar tissues in the diabetic rats (group 2) were irregular in shape compared to normal rats (group 1), however 50% of the scar in rats wound treated with aqueous cream containing 10% mushroom extract was in straight line (Plate 4.3). Overall the scar tissues in diabetic rats were bigger compared to normal rats.

4.3.4 Histological Analysis of Healed Wounds

Histological analysis to evaluate the morphological changes in healed wound tissue to further confirm the effect of the aqueous extract of *G. lucidum* on wound healing in normal and diabetic rats. Evidence of repair in 16 day specimens in normal rats (group 1) are depicted in Plate 4.4.

In normal rats (Group 1), the wound healing is seen primarily in the dermis, the epithelium over the healed wound is intact, and is indistinguishable from the surrounding epithelium on all groups (Plate 4.4). Skin layer were clearly identified, epidermis and dermis were observed to be normal. A significant number of fibrils or fibroblasts were also observed in all treatment groups. Inflammatory cells are scant within the wound and new capillary formation near the wound site is not striking. Moderate variation is seen among the groups treated with aqueous cream, Intrasite gel and aqueous cream containing mushroom extract at 16-day post-operation, however there is a general pattern.

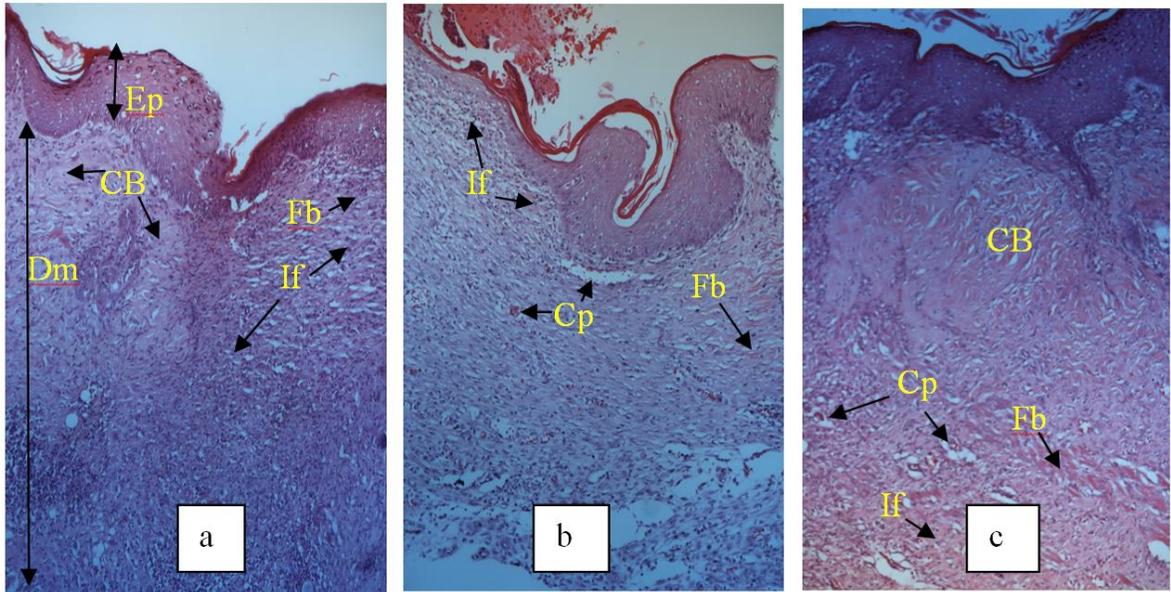


Plate 4.4: Micrograph of wounds in normal rats (Group 1) at day 16 post-operation (H & E staining; 10x magnification). (a): specimen of wound treated with aqueous cream (negative control); (b): specimen of wound treated with Intrasisite gel; (c): specimen of wound treated with aqueous cream containing 10% mushroom extract. Well defined epidermis and dermis layer (Ep, Dm), significant numbers of fibroblast cells (Fb) parallel to epidermis, and fewer inflammatory cells (If), new capillary formation (Cp) and collagen bundle (CB) can be seen in all groups in normal rats.

Directly under the epithelium, in wounds of normal rats, is located a wide area of fibrosis in which newly-formed, fine fibres are located parallel to the epithelium. Deeper in the dermis a more organized fibre-pattern occurs, although randomly-oriented fibres are plentiful. Large collagen bundles can be seen, are surrounded by circumferential fibrils (Plate 4.4). Deeper in the dermis we observed dense connective tissue layer followed by loose connective tissue layer underneath the epidermis layer with well organised collagen fibres as shown in plate 4.4 (b) and (c).

Histological analysis of healed wound specimen at day 16 post-operation in diabetic rats is depicted in Plate 4.5. Wounds of negative control in Plate 4.5 (a) and positive control groups in Plate 4.5 (b) in the diabetic rats, the inflammatory cells were abundant around the wound site but detailed structure could not be identified. Cell response was observed not to

be proper at deeper layer and epithelial tissues and collagen fibres were irregular. Neutrophilic infiltrations were present at the dermal surface and early development of scar tissue was observed. Although the epidermis could not be observed under the wound scab in negative control group, infiltrations were observed; irregular myofibroblast distributions, collagen fibres and many fibroblasts were present as shown in Plate 4.5 (a).

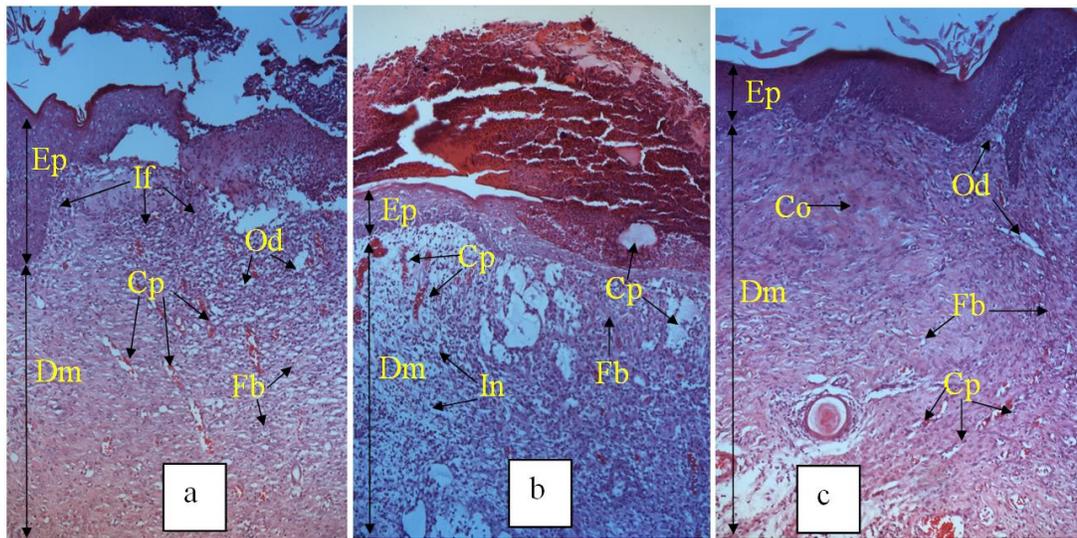


Plate 4.5: Micrograph of wounds in Type-1 diabetic rats (Group 2) at day 16 post-operation (H & E staining; 10x magnification), showing epidermis and dermis layer (Ep, Dm), migrating fibroblast cells (Fb), inflammatory cells (If), oedema cells (Od), new capillary formation (Cp) and collagen fibres (Cf). (a): specimen of wound treated with aqueous cream (negative control); (b): specimen of wound treated with Intracite gel; (c): specimen of wound treated with aqueous cream containing 10% mushroom extract.

The continued of fibroblastic activity, wound scab and invasive inflammatory cell infiltrations underneath were also noticed in positive control group as shown in Plate 4.5 (b). In group treated with aqueous cream containing 10% (w/w) mushroom extract, the epithelium over the healed wound is intact, and is indistinguishable from the surrounding epithelium as shown in Plate 4.5 (c). Skin layer were clearly identified, epidermis and dermis were observed to be normal as in normal rats (Group 1). A large number of fibrils or

fibroblasts, lesser inflammatory cells and new capillary formation near the wound site can be seen. The histological results indicated and supported that the healing in the mushroom extract treatment group was better and more rapid when compared with the negative and positive control groups of diabetic rats.

For the analysis of collagen matrix in the healed wound of diabetic rats on day 16 post-operation, sections of specimen were stained with Masson Trichrome staining method. Histological analysis of healed wounds in normal rats is depicted in Plate 4.6, revealed that wounds dressed with 10% (w/w) aqueous extract of *G. lucidum* showed excellent epithelisation and well-formed granulation tissue, markedly fewer inflammatory cells, more collagen (stained in blue) accompanied with angiogenesis (Plate 4.6).

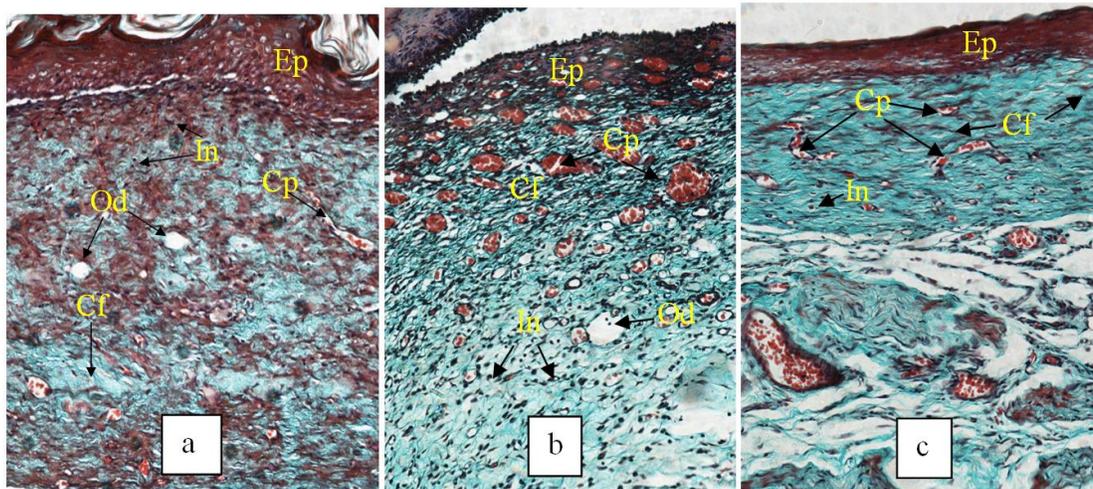


Plate 4.6: Micrograph of wounds in Type-1 diabetic rats (Group 2) at day 16 post-operation (Masson Trichrome staining; 20x magnification). Ep: epithelium cells; If: inflammatory cells; Od: oedema; Cp: blood capillary and Cf: collagen fibre. (a): specimen of wound treated with aqueous cream (negative control); (b): specimen of wound treated with Intracite gel; (c): specimen of wound treated with aqueous cream containing 10% mushroom extract.

In contrast wounds in negative and positive control groups of diabetic rats, newly formed epithelium cells was not firmly attached to the dermis and hemorrhagic scab can be seen in the Intracite treated group. Extracellular matrix contained abundant plasma and

inflammatory cells, poorly formed capillary vessels and lesser collagen matrix was observed in positive control group treated with aqueous cream. Plentiful of blood vessel can be seen in the wound treated with Intrasite gel, actively supply oxygen and nutrient to the wound site indicated that they are still in the reparative stage. More inflammatory cells and less collagen accompanied by swollen endothelial cell and poorly formed capillary channels, and evident haemorrhage as shown in Plate 4.6 (a). A significant reduction of granulation tissue formation and incomplete matrix maturation and remodelling, characterized by loose connective tissue in an irregular fashion, prominent oedema and haemorrhage was shown in group treated with Intrasite gel as shown in Plate 4.6 (b). Well-formed collagen matrix with scattered newly formed capillary vessels lined by a single layer of endothelial cells showing good re-epithelialization and well-formed granulation tissue was shown in group treated with cream containing 10% (w/w) mushroom extract. Spindle-shaped and oval fibroblasts are oriented parallel to the epithelial surface. Neovascularization is characterized by well-organized capillary vessels and absence of haemorrhage crust as shown in Plate 4.6 (c).

Scar width and blood vessel count was performed at the healed wound area. The scar tissue was very prominent in all groups in the diabetic rats showing the scar gap at wound closure (Plate 4.7).

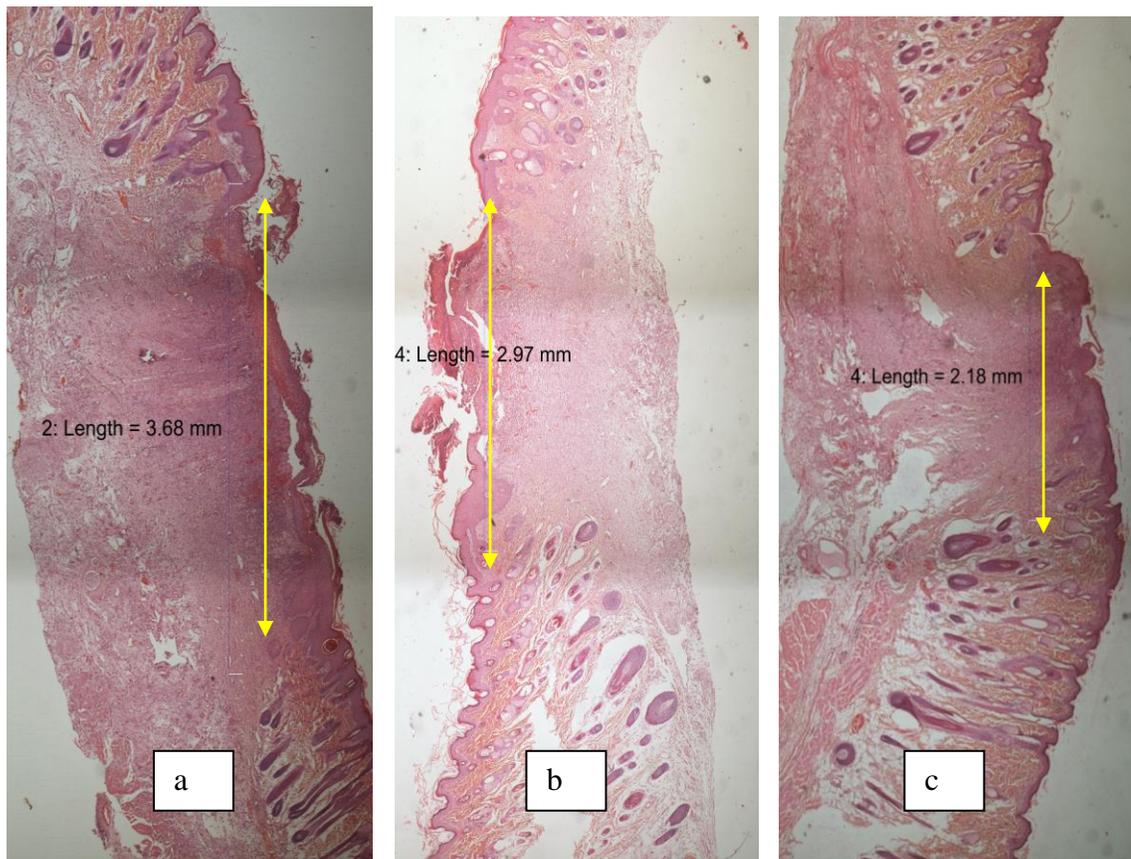


Plate 4.7: Micrograph showing scar width on day 16 post-operation (H & E staining; 4x magnification). (a): wound treated with aqueous cream; (b): wound treated with Intrasite gel; (c): wound treated with aqueous cream containing 10% (w/w) mushroom extract

The measurements for the scar-width of healed wound was 2.56 ± 0.59 mm, 3.42 ± 0.75 mm and 2.91 ± 1.06 mm for rats treated with aqueous cream containing mushroom extract, aqueous cream alone and Intrasite gel respectively. Meanwhile the blood vessel count for the three treatment groups was 77 ± 23.65 , 68.25 ± 12.58 and 51.40 ± 16.88 ; respectively. Group of diabetic rats treated with aqueous cream showed widest gap, followed by Intrasite gel moderate and aqueous cream containing 10% (w/w) of mushroom extract is smaller (Plate 4.7).

4.3.5 Effects of *G. lucidum* on *in vivo* Antioxidant Capacity and Oxidative Stress Indices in Normal and Diabetic Rats.

The antioxidant capacity and oxidative indices in experimental rats can be related to wound healing in normal and diabetic rats. *In vivo* antioxidant capacity and oxidative damages during wound healing and STZ-induction was evaluated in the serum of rats on day 16 post operation.

4.3.5.1 Effect of *G. lucidum* on *in vivo* Antioxidant Capacity in the Serum of Experimental Rats.

In vivo cupric ion reducing antioxidant capacity (CUPRAC, Apak *et al.*, 2004) were used to measure the combined antioxidant effect of the non-enzymatic defences in biological fluids which may be useful in providing an index of ability to resist oxidative damage (Halliwell *et al.*, 1995). The results in Fig. 4.4 showed CUPRAC values in normal and diabetic rats.

The CUPRAC values in normal rats wound treated with Intrasite gel was the lowest ($p < 0.05$) among the groups (Group 1), which was (0.09 ± 0.01) , followed by negative control rats treated with aqueous cream which was (0.13 ± 0.01) , aqueous cream containing 10%, 5%, 15% and 20% (w/w) mushroom extracts which were (0.24 ± 0.02) , (0.24 ± 0.04) , (0.25 ± 0.02) and (0.25 ± 0.02) respectively. CUPRAC levels in normal rats treated with aqueous cream containing 5%, 10%, 15% and 20% (w/w) extract of *G. lucidum* were significantly ($p < 0.05$) elevated after 16 days of application compared to control. This indicated that Intrasite gel did not contribute or trigger the *in vivo* antioxidant capacity of normal rats whereas aqueous cream containing mushroom extract act as exogenous

antioxidant that absorbed through the wound capillary into the blood or able to trigger the mechanism of endogenous antioxidant activity of the normal rats due to oxidative stress.

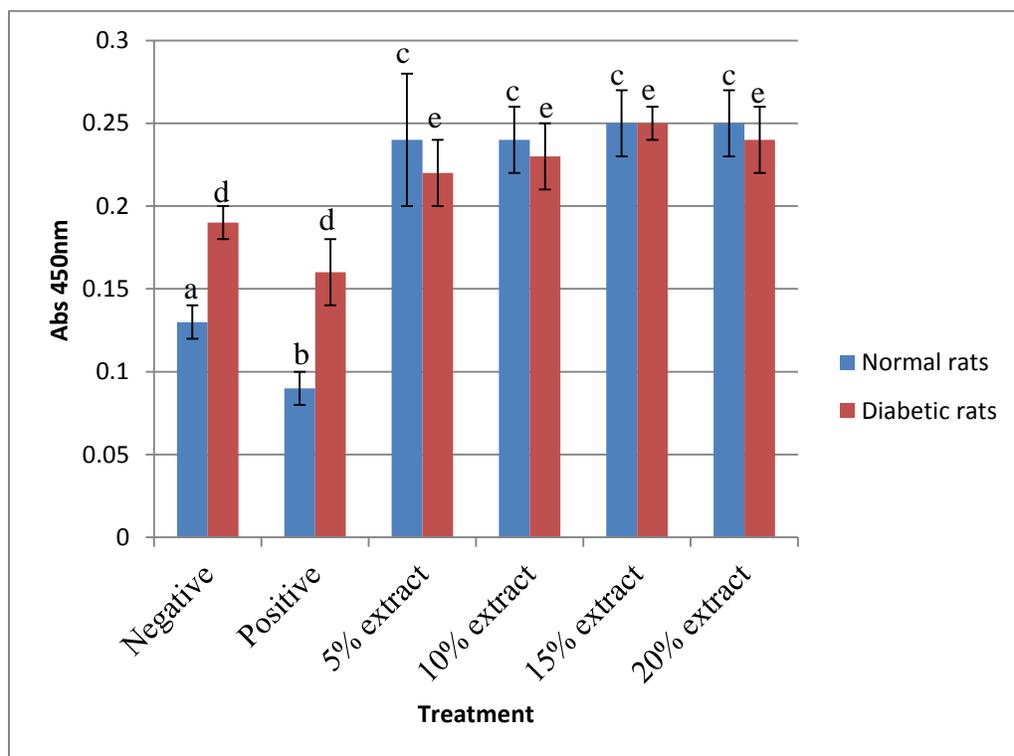


Figure 4.4: Non-enzymatic oxidation using *In vivo* cupric ion reducing antioxidant capacity (CUPRAC) value at day 16 post-operation for normal and diabetic rats. Data are expressed as means \pm standard error (n=6). Means with different alphabets (a, b, c, d & e) were significantly different ($p < 0.05$) by ANOVA and Duncan's multiple range tests. Extract: Aqueous cream containing 5%, 10%, 15% and 20% of *G. lucidum* extract.

From the results of CUPRAC levels in diabetic rats (Fig. 4.4), showed that wound treated with Intrasite gel was the lowest among the groups (Group 2), which was (0.16 ± 0.02) whereas diabetic rats wound treated with aqueous cream containing 5%, 10%, 15% and 20% (w/w) extract of *G. lucidum* were significantly ($p < 0.05$) elevated compared to control, which was (0.22 ± 0.16), (0.23 ± 0.02), (0.25 ± 0.01) and (0.24 ± 0.02) respectively. The results revealed that topical application of the cream containing mushroom extract significantly ($p < 0.05$) elevated the endogenous and exogenous antioxidant capacity in the experimental rats. This indicated that aqueous cream containing mushroom extract

contributed or triggered to resist the oxidative damage in the diabetic wound healing of Group 2 rats.

4.3.5.2 Effect of *G. lucidum* on Advance Oxidative Protein Products (AOPPs) Levels in the Serum of Experimental Rats.

Measurement of AOPP is a reliable marker to estimate the degree of oxidant-mediated protein damage and to predict the potential efficacy of therapeutic strategies aimed at reducing such an oxidative stress (Witka-Sarsat *et al.*, 1996). The AOPPs at day 16 post-operation in normal and diabetic rat are depicted in Figure 4.5 and data was derived from the AOPP standard curve given in Appendix A, viii, pp 136.

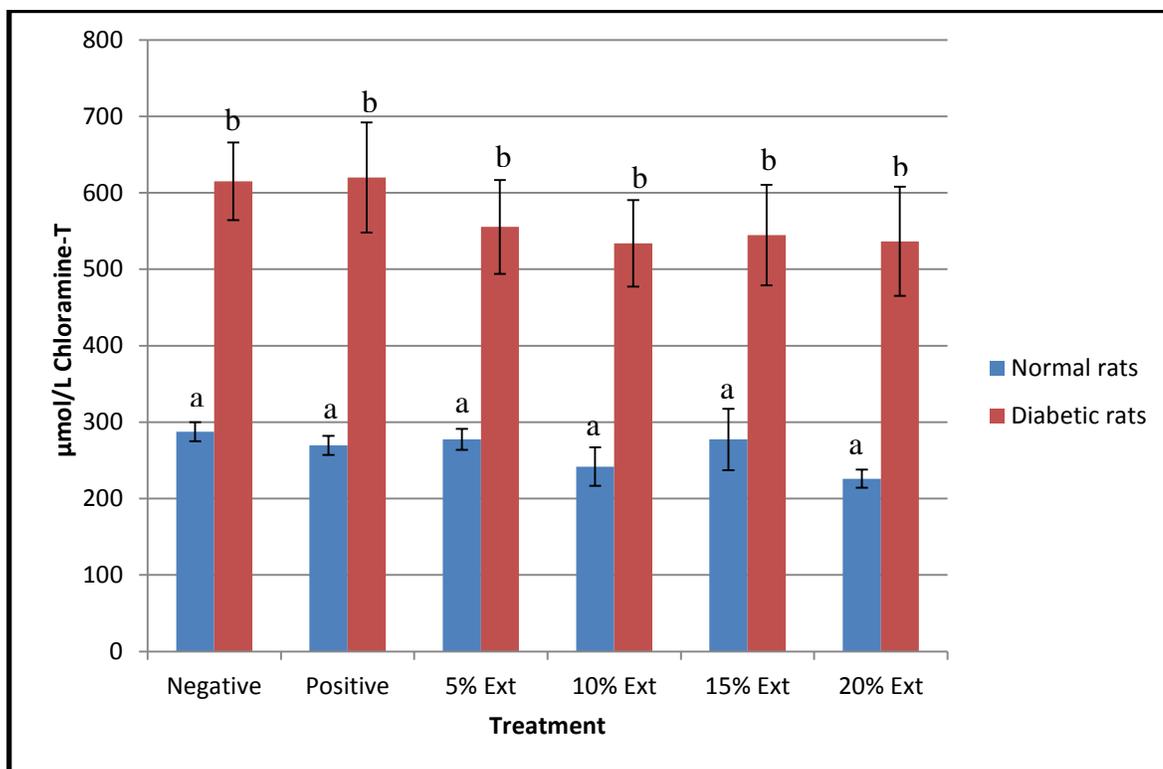


Figure 4.5: Advanced Oxidation Protein Products (AOPPs) at day 16 post-operation for normal and diabetic rats. Data are expressed as means \pm standard error (n=6). Means with different alphabets (a & b) were significantly different ($p < 0.05$) by ANOVA and Duncan's multiple range tests. Extract: Aqueous cream containing 5%, 10%, 15% and 20% of *G. lucidum* extract.

The results in Figure 4.5 showed that no significant differences ($p < 0.05$) in AOPP values among all the treatment groups in normal rats (Group 1). The advanced oxidation protein products values were (287.50 ± 12.44) , (269.67 ± 12.52) , (277.33 ± 13.67) , (277.11 ± 40.14) , (266.00 ± 11.68) and (241.83 ± 25.10) , in normal rats treated with aqueous cream (negative control), Intracite gel (positive control), aqueous cream containing 5%, 15%, 20% and 10% (w/w) mushroom extract. This indicated that topical application of cream containing mushroom extract did not significantly ($p < 0.05$) alter the AOPP values in normal rats at day 16 post-operation.

In diabetic rats, the AOPPs values of rats treated aqueous cream, Intracite gel and aqueous cream containing 5%, 10%, 15% and 20% (w/w) mushroom extract was (615.17 ± 50.77) , (620.00 ± 71.94) , (555.33 ± 61.50) , (533.67 ± 56.60) , (544.67 ± 65.75) and (536.50 ± 71.29) respectively. No significant changes in AOPPs values in the groups treated with mushroom extract compared to negative and positive control groups in diabetic rats, but a slight reduction in AOPPs was seen in all groups treated with cream containing mushroom extract compared to negative control group. However, AOPP values were significantly ($p < 0.05$) higher in diabetic rats (Group 2) compared to normal rats (Group 1), which may be due to the untreated diabetes condition or high glucose levels in diabetic rats during the whole period of experiment.

4.3.5.3 Effect of *G. lucidum* on Lipid Hydroperoxide (LHP) Levels in the Serum of Experimental Rats.

Lipids are susceptible to oxidation and LHP is the product of lipid oxidation, which may serve as the oxidation biomarker. Lipid hydroperoxide values were expressed as $\mu\text{mol/L}$ of TEP equivalents according to Esterbauer and Chessemann (1990). Lipid

hydroperoxide data were derived from the LHP standard curve given in Appendix A, vii, pp 134. Lipid hydroperoxide values at day 16 post-operation in normal and diabetic rats are shown in Figure 4.6.

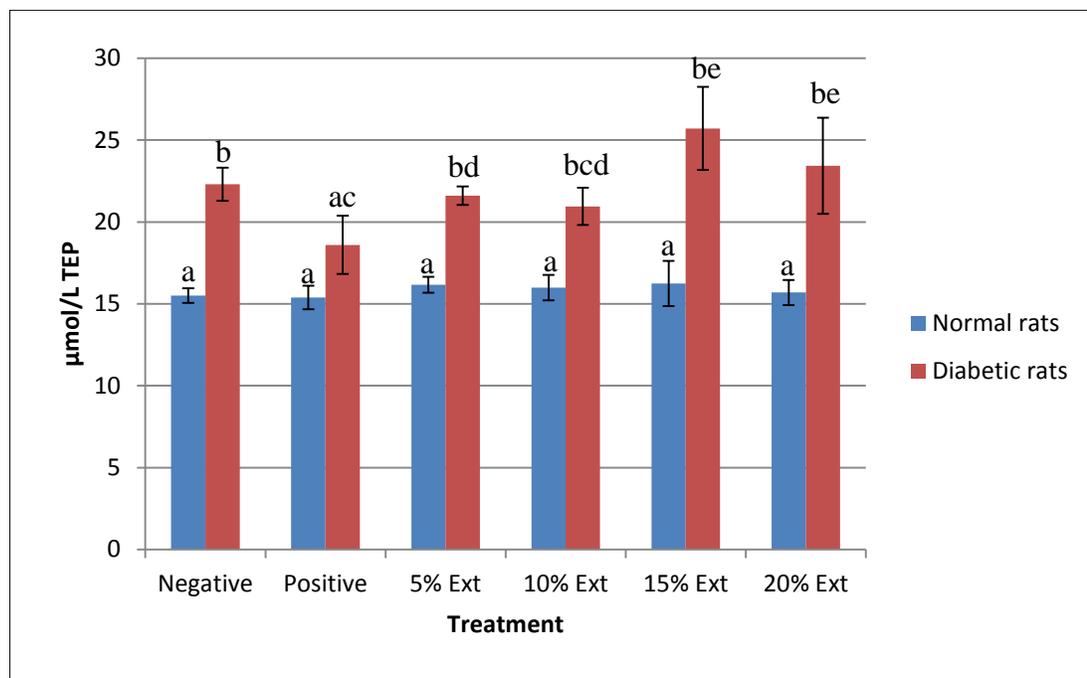


Figure 4.6: Lipid Hydroperoxides Product (LHP) values in normal and diabetic rats. Data are expressed as means \pm standard error (n=6). Means with different alphabets (a, b, c, d & e) were significantly different ($p < 0.05$) by ANOVA and Duncan's multiple range tests. Extract: Aqueous cream containing 5%, 10%, 15% and 20% of *G. lucidum* extract.

The results showed that LHP values in the groups treated with aqueous cream, Intracite gel, aqueous cream containing 5%, 10%, 15% and 20% (w/w) mushroom extracts are (15.51 \pm 0.45), (15.38 \pm 0.72), (16.16 \pm 0.48), (15.99 \pm 0.77), (16.25 \pm 1.38) and (15.69 \pm 0.76). No significant changes in all the groups in normal rats. This indicated that topical application of cream containing mushroom extract did not contribute to the LHP production or lipid oxidation in the normal rats at day 16 post-operation.

In diabetic rats (Fig. 4.6), the LHP values in the groups treated with aqueous cream, Intracite gel, aqueous cream containing 5%, 10%, 15% and 20% (w/w) mushroom extracts are (22.30 \pm 1.01), (18.60 \pm 1.78), (21.60 \pm 0.56), (20.90 \pm 1.14), (25.71 \pm 2.54), and (23.43

± 2.94), respectively. No significant differences ($p < 0.05$) in LHP values in the groups treated with mushroom extract compared to negative control group, but LHP values in the groups treated with mushroom extract were significant higher when compared to positive control group. Notably, group treated with aqueous cream containing 10% mushroom extract was comparable to positive control group which has the lowest LHP value. However, the result in the LHP did not correlate with the concentration of the mushroom extract, healing time and the blood glucose reading in diabetic groups. This indicated that topical application of cream containing mushroom extract did not contribute to the LHP production or lipid oxidation in the diabetic rats at day 16 post-operation. However, LHP values was significantly ($p < 0.05$) higher in diabetic rats (Group 2) compared to normal rats (Group 1), which may be due to the oxidative damage caused by the untreated diabetes during the whole period of experiment.

5. DISCUSSIONS AND CONCLUSIONS

5.1 Streptozotocin-Induced (Type-1) Diabetes Animal Model

Streptozotocin (STZ) selectively destroys pancreatic β -cells, inhibits the syntheses and release of insulin, and causes the onset of diabetes mellitus (Elias *et al.*, 1994). It has been shown that blood glucose levels peak at 1 to 3 days after a single high dose injection of STZ, and then remains elevated. STZ-induced diabetes in rodents is considered to be a model of insulin-dependent diabetes mellitus, and is widely used in the study of insulinopenia and hyperglycaemia (Rees & Alcolado, 2005). Diabetes that has been induced by a single high dose of STZ is typically accompanied by diabetic symptoms such as weight loss, polyuria, hyperglycemia, and neuroendocrine dysfunction (Elias *et al.*, 1994). Impaired wound healing occurs in patients with diabetes, and it has been reported to be associated with high blood glucose levels (Hoogwerf, 2001). Hence, in the present study, STZ induced diabetic rats were used as the model of diabetes to study diabetic wound healing.

Experimental and clinical studies have demonstrated that control of blood glucose is significant in the diminution of post-operative morbidities, including wound infection in patients with either Type-1 or Type-2 diabetes (Hoogwerf, 2001). Topical application of aqueous extract of *G. lucidum* into wounds did not affect the blood glucose levels of diabetic rats in this study, thus, it seems unlikely that the restoration of blood glucose contributes to the effect of mushroom extract on diabetic wound healing. STZ induced diabetes is considered to be a model of Type-1 diabetes (i.e., insulin dependent) conditions.

Since to date, no effective approach has been found that control both hyperglycemia and resolve the issue of impaired wound healing, the study might be beneficial to patients with Type-2 diabetes (Rees & Alcolado, 2005).

5.2 Wound Healing Effect of Aqueous Extract of *G. lucidum*

Wound healing, or wound repair, is the body's natural process of regenerating dermal and epidermal tissue, involving inflammation, haemostasis, fibroplasia, collagen deposition, neovascularization, epithelialisation and remodelling, which fall in three major phases; inflammation, proliferation, and maturation (Abdulla *et al.*, 2010; Brem & Tomic-Canic, 2007). After initial wounding, blood extravasation causes platelet aggregation and blood clotting. These events initiate inflammation and set the stage for repair processes. Throughout the repair phase, the provisional wound matrix is remodelled and replaced with scar tissue, made up of new collagen fibres, elastin fibres, and proteoglycans which partially bring back the structure and function of the tissue (Martin, 1997). This is accomplished by the migration, proliferation and differentiation of epithelial cells, vascular endothelial cells and dermal fibroblasts from adjacent uninjured tissue to the wound site (Clark, 1993). Finally, the injured tissue is repaired rather than regenerated (Abdulla *et al.*, 2010; Martin, 1997).

Re-epithelialisation period is the indication of the period wound healing occurs with a thin layer of epithelial cell formation. It is basically dependent on the size of the wound and factors affecting the healing process, like microbial infection and pathological complication of an individual (hypertension or diabetes). Wound closure is the biomechanical phenomenon in which the wound boundaries are drawn towards the center of wound. By reducing the size of the defect, wound contraction is usually beneficial to the

overall repair process. However, insufficient contraction may cause delayed or impaired healing, whilst excess contraction often induces poor quality repair with substantial scarring (Rudolph *et al.*, 1992; Shah *et al.*, 1992).

The main concern with diabetic wounds is poor or delayed healing. Healing difficulties are caused by the peripheral arterial diseases and peripheral neuropathy that can occur with diabetes, wherein the small blood vessels in various parts of the body mostly in the extremities (hands and feet) grow narrower and lower the blood circulation to those areas. A shortage of circulation in the extremities can result in a reduced supply of oxygen and nutrients to the body tissue and nerves.

In this study we used streptozotocin-induced model in diabetic groups. Streptozotocin-induced rodent is a model widely used in the study of insulin-dependent diabetes mellitus and hyperglycemia (Rees & Alcolado, 2005). From the results of re-epithelialisation and rate of wound closure, the extract of *G. lucidum* showed comparable wound healing effects as in commercial Intrasite gel. The mechanism of wound healing in healthy rats is well guided and it is through integration of multiple signals in the form of cytokines and chemokines released by keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets (Greenhalg, 2003). The result revealed that extract of *G. lucidum* did not significantly change the process of wound repair in normal rats, which is probably due to the intrinsic immunity and endogenous antioxidant defences system in the normal rats work well.

However, the impaired wound healing in acute wounds of diabetes rats, possibly due to a decreased fibroblast proliferation (Black *et al.*, 2003), delayed cellular infiltration, lack of granulation tissue formation, decreased collagen matrix, reduced angiogenesis, and its organisation (Greenhalg, 2003). The wounds of diabetic animals were associated with an

impaired inflammatory response, reduced deposition of collagen matrix and scattered newly formed capillary vessel in deep dermis (Plate 4.5). This was observed in the delayed of contraction in wounds area at day 4 to day 12 after treatment in diabetic animals (Fig. 4.3). The similar observation was reported by Hehenberger *et al.*, (2000) that fibroblasts cultured from chronic wounds, in particular from diabetic foot ulcers demonstrated impaired proliferation of fibroblast. The similar effect of impaired collagen deposition and fibroblast proliferation were observed in the microscopic analysis of healed wounds tissue treated with aqueous extract alone (negative control group). The effects of diabetes on healing are diverse, multi-factorial, complex and inter-related. It is one of the well-known intrinsic factors which affect wound healing. Based on the results in Plate 4.5, diabetes affects almost all stages of wound healing to some extent. Lowered healing capacity in diabetes is the result of several factors, including free radical generation, elevated blood glucose levels, local ischemia, and suppressed cell-mediated immunity (Senel *et al.*, 1997). The underlying mechanisms are not fully investigated.

As indicated by our results, wound healing on day 12 to day 16 (Fig. 4.2, 4.3) might enter the most active stage involving inflammation and cell proliferation, where fibroblast proliferated at peak and was responsible for initiating angiogenesis, collagen formation and epithelialisation. Aqueous extract of *G. lucidum* may stimulate all the responses at various stages of wound healing, including inflammation, haemostasis, proliferation, and remodelling.

The componential analysis showed that the aqueous extract of *G. lucidum* contained 25% polysaccharides, 0.45% ganoderic acid A and 0.069% adenosine (Table 4.1). In the study we used the aqueous extract of *G. lucidum* as a whole and the marker compounds was determined. There is a tendency to expect that if a compound is found to

be the “active” component of a natural material, it must be more effective if isolated and concentrated in pure form (Schaich *et al.*, 2005). When multiple components are synergistic, enhancing and complementary reaction mechanisms, it may be more effective in whole foods and extracts alone. In contrast, when multiple components are competitive, fighting for the same assay substrates and binding sites, activity of individual compounds increases with isolation (Schaich *et al.*, 2005). Fractionation often results in loss of activity, or less increase per fraction than might be expected. This may be due to decomposition of the active products breakup of synergistic relationships (Schaich *et al.*, 2005). The aqueous extract of *G. lucidum* may be useful as starting materials for the development of chemical therapeutic agents in treatment of chronic wound healing. However the exact mechanisms of action of the polysaccharides complex and triterpenes remain to be fully understood.

The effect of polysaccharides on wound healing was reported by Bae *et al.*, (2005), that the polysaccharides isolated from *Phellinus gilvus* (Schw.) Patouillar (mustard-yellow polypore) enhanced dermal wound healing in normal (Bae *et al.*, 2005b) and streptozotocin-induced diabetic rats (Bae *et al.*, 2005a). The pharmacological studies focused on the wound healing promoting effect of mushroom’s polysaccharides are rather scarce. These results are in agreement with our study that hot water extraction (100%) is an appropriate method for polysaccharide extraction in mushroom. Kwon *et al.*, (2009) also reported that the β -glucan purified from medicinal mushroom *Sparassis crispa* (cauliflower mushroom) increased macrophage infiltration into the wound tissue and enhanced wound healing. Accordingly, the mechanism of β -glucan-induced wound healing was associated with increased type I and III collagen biosynthesis. However, while the β -glucan was orally administered to the rats in the study of Kwon *et al.*, (2009) our experimental rats were topically treated with aqueous cream containing varying concentration of *G. lucidum*

extracts. Most recently, polysaccharides purified from *Tremella fuciformis* (white jelly mushroom) and *Auricularia auricula* (wood ear mushroom) were shown to enhance wound healing using the *ex vivo* porcine skin wound healing model (Khamlue *et al.*, 2012). The water-soluble polysaccharide fractions of *G. lucidum* have been reported to have wound healing effects especially against ulcer lesions (Gao *et al.*, 2002; Gao *et al.*, 2004). Elsewhere, Sun *et al.*, (2011) reported that *G. lucidum* polysaccharides showed wound healing effects on intestinal epithelium using a non-transformed small-intestinal epithelial cell line, IEC-6 cells.

Macrophage activities stimulated by β -glucans may also benefit wound healing and reduce scar tissue levels after surgery or trauma, as revealed by both animal and human studies on *Saccharomyces cerevisiae* (Mayell, 2001; Portera *et al.*, 1997). In normal human dermal fibroblasts this β -glucan preparation stimulated procollagen mRNA and collagen biosynthesis, together with increased NF-1 (Wei *et al.*, 2002b). Inhibition of NF-1 by pentifylline blocked induction of procollagen mRNA by the same β -glucan, which also induced mRNA synthesis of many other wound growth factors including activator protein-1, specificity protein-1, neurotrophin 3, platelet-derived growth factor A, B, fibroblast growth factor acidic, fibroblast growth factor basic, transforming growth factor a, b, and vascular endothelial growth factor (Wei *et al.*, 2002a). From the multiple research papers coated above, the possible target compound structure could be β -glucan that present in *G. lucidum*. However, in order to unravel the possible mechanism involved in the process of wound healing either by regulating body's defensive mechanism or by direct action of mushroom extract on wound, more work is required.

Free radical scavenging activity could be a major mechanism by which *G. lucidum* products protect against cellular damage (Moseley *et al.*, 2004). Based on the study, *G.*

lucidum may act at various levels in the immune mechanism, such as antibody production, release of mediators and tissue responses to these mediators in the target area in modulating the immune responses (Moseley *et al.*, 2004). Inhibition of growth of pathogenic microorganisms was observed in rats treated with mushroom extract. Our experiment revealed that healing of the wound by aqueous cream formulated with 10% extract of *G. lucidum* was comparable to the commercial Intrasite gel in normal and diabetic animals.

5.3 Macroscopic and Microscopic Analysis of Healed Wounds

5.3.1 Macroscopic Analysis of Healed Wounds

Wound microenvironment is one of the factors contributed to healing process. To comprehend the microenvironment of chronic and acute wounds, the pathophysiology of impaired wound healing has been increasingly investigated. Raised levels of matrix metalloproteinases (MMPs) result in excessive proteolysis of tissue, as well as of growth factors and their receptors (Lobmann *et al.*, 2002). Matrix metalloproteinases are responsible for controlled degradation of the extracellular matrix as well as migration in normal wound healing. They also affect angiogenesis and remodelling of the dermis (Trenegove *et al.*, 1999). From the macroscopic analysis at day 4, 8, 12 and 16 post-operation (Plate 4.2 and 4.3), wounds of rats treated with aqueous cream containing mushroom extract was moist, preventing the cell at wound site from drying and death during the period of wound healing. The fluids at the wound sites may be an important reservoir of growth factors that promote the healing process (Fu *et al.*, 1998). Growth factors are the polypeptides that control the growth, metabolism and differentiation of cells and regulate the process of tissue repair (Fu *et al.*, 1998). Growth factors bind to specific high-affinity receptors on the cell-surface to stimulate cell growth. Although they are

present in small amounts, they exert a powerful influence on the process of wound repair (Celebi *et al.*, 2002). Some studies reported dealing with growth factors in burn wound healing, in which it has been suggested that growth factors play an important role in the healing process (Fu *et al.*, 1998). Several defined peptide growth factors, including epidermal growth factor (EGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and transforming growth factor-beta (TGF- β), have been shown to stimulate cellular proliferation and synthesis of the extracellular matrix (Robson *et al.*, 1998). However, in order to unravel the possible mechanism involved in the process of wound healing by regulation of the growth factors stimulated by mushroom extract, more work is required.

The observation indicated that the maximum healing effect was in the wounds treated with cream containing 10% mushroom extract (Plate 4.2). The appearance of wound surface is absence of haemorrhagic crust, shorter healing time with steady wound contraction during the process of healing were present in group treated with 10% mushroom extract. In conclusion, when the results were evaluated, it was determined that mushroom extract-containing formulations are effective in the wound healing process. The wound healing activity indicated by application of aqueous cream containing mushroom extract could be partly due to the base cream in the formulation. Aqueous cream is a light, non-greasy moisturiser cream that is made from a mixture of emulsifying ointment (which contains paraffin oils) and water, with phenoxyethanol as antimicrobial preservative. It helps to prevent dehydration of stratum corneum, which is the outer layer of skin cells, reduces cracked, scaly and itchiness due to dry skin. Due to the moisturising effect of aqueous cream, it provides good environment for wound healing. In contrast, Intrasite gel is a clear amorphous hydrogel containing a modified carboxymethyl cellulose polymer, water,

and propylene glycol. Intrasite gel is indicated for the removal of non-viable tissue from shallow, undermined and deep wound leg pressure sores, leg ulcers, diabetic foot ulcers, malignant wounds, burns, surgical wounds, scalds, lacerations, grazes, amputations and fungating ulcers. It absorbs exudates and leaves the wound dry, therefore promote rapid contraction of wound but newly formed granulation tissue was desiccated and subsequently died, However, the effect of cream containing mushroom extract play an important role in wound closure will be further confirmed by the histological evaluation of healed wound sections.

5.3.2 Microscopic Analysis of Wounds Section

Tissue injury resulting in irreversible tissue loss and initiates the repair process. The repair of dermal loss is by scarring, where a new cell population occupies in a new connective tissue matrix (Ehrlich & Kerummel, 1996). The chemical composition of a scar may be similar to normal dermis, but the arrangement of that tissue is altered. The lack of ability of the organism to reassemble collagen into a normal dermal pattern is an attribute of a scar, but in many cases it restores normal function. With impaired healing process in diabetic rats, wound healing delayed or chronic wounds arise, while the overproduction of scar tissue results in keloid or hypertrophic scarring. The maturation of scar depends on the character of the quality of deposited connective tissue, its resident cell populations, and the interactions between those components (Ehrlich & Kerummel, 1996). The objective in wound management is to heal the wound in the shortest time possible, with minimal discomfort, pain nor scarring to the patient. A flexible and fine scar with functional restore at the site of wound closure is desired for patients.

In the normal rats, epidermal regeneration and remodelling of the dermis was organised in group treated with the extract compared to negative control treated with vehicle (Plate 4.4). Newly formed capillary vessels were disposed vertically toward the wound surface and were identical to those of normal dermis. On the other hand, in diabetic rats treated with the extract, re-epithelization was moderate to complete with epidermal elongation spreading over the wound surface. Dermal restoration was characterized by granulation tissue rich in fibroblasts, normally oriented parallel to the epidermal layer. An average amount of collagen fibrils and collagen bundles were organized in a more regular fashion than that seen in the diabetic rats treated with aqueous cream containing. Newly formed capillary vessels were observed in moderate numbers in the dermis of the entire wound area. Abnormalities associated with diabetic wounds include, increased levels of proteinases, impaired neovascularization, prolonged inflammation, decreased synthesis of collagen, and defective macrophage function (Ehrlich & Kerummel, 1996).

During inflammation phase, macrophages and neutrophils are recruited to the wound site (Maruyama *et al.*, 2007). Macrophages will engulf debris cells and produce factors such as vascular endothelial growth factor (VEGF) -A and -C which regulate tissue repair and stimulate the formation of lymphatic and blood vessels in the wound site (Maruyama *et al.*, 2007). In wounds of non-diabetic rats, lymphatic vessels are formed to maintain normal tissue pressure through the drainage of lymph from interstitial spaces and facilitating the movement of cells that mediate immune response (Maruyama *et al.*, 2007). However, in diabetic rats, the delayed in lymphatic vessels formation leads to edema and delayed removal of debris and infiltration of inflammatory cells (Plate 4.5). In a study that experiments the relationship between glucose levels and macrophage function, it was found that the wound healing was delayed due to impaired macrophages response and low level of

VEGF (Maruyama *et al.*, 2007). Histology confirmed and explained the macroscopical differences in wound healing between normal and diabetic rats.

Histological studied revealed that animals treated with extract of *G. lucidum* show angiogenesis in granulation tissues, thus improves circulation to the wound site, and providing oxygen and nutrients essential for the healing process (Szabo *et al.*, 1995). Re-epithelization was faster in the group treated with extract of *G. lucidum* incorporated creams. The extract could stimulate epithelial cell proliferation and angiogenesis in the healing process. The overall results showed that aqueous extract of *G. lucidum* increase cellular proliferation and collagen synthesis and promote wound healing in diabetic rats.

5.4 Oxidative Status of Rats during Wound Healing

Several types of reactive species are generated in the body as a result of metabolic reactions in the form of free radicals or non-radicals. These reactive species may be either oxygen derived or nitrogen derived and called pro-oxidants. They attack macromolecules including lipid, protein, DNA etc. causing tissue/cellular damage. To counter their consequence, the body is endowed with another category of compounds called antioxidants. These antioxidants are constructed either endogenously or received from exogenous sources and include enzymes like catalase, superoxide dismutase, glutathione reductase, and glutathione peroxidase, minerals like Mn, Cu, Se, and Zn, and vitamins like vitamin A, C and E. Other compounds with antioxidant activity include flavonoids, glutathione, uric acid, and bilirubin etc. In a healthy body, pro-oxidants and antioxidants maintain a ratio and a shift in this ratio towards pro-oxidants gives rise to oxidative stress (Irshad & Chaudhuri, 2002).

Lipids are quite susceptible to oxidation and their oxidation products may serve as appropriate biomarkers. The reactivity of lipid hydroperoxides is such that they are damaging to proteins within tissues. Lipid hydroperoxides are the important biomarkers to measure or assess the extent of oxidative stress *in vivo*. In our study, LHP value were not significantly ($p < 0.05$) altered mushroom extract incorporated cream in both normal and diabetic groups (Fig. 4.6). However, we observed that the LHP values were slightly higher in group treated with cream containing mushroom extract, in which we believe that it may due to STZ injection; cause increased glucose levels during prolonged period of experiment without any medication to control the diabetic condition. Overall results showed that the LHP values in diabetic groups were higher than the normal groups without STZ injection. This indicated that the metabolic abnormalities of diabetes cause lipids oxidation in the targeted organ, leading to changes in cellular structure and function of the experimental diabetic animals (Dana & David, 2005).

Oxidative damage of proteins is one of the modifications leading to severe failure of biological functions and cell death. Continuing exposure of protein to reactive molecules leads to spontaneous post-synthetic modifications and oxidation to form advanced oxidation protein products (Witko-Sarsat *et al.*, 1996). Natural antioxidants are being extensively studied for their capacity to protect organisms and cells from damage brought by oxidation and degenerative diseases (Cazzi, 1997). Previous study showed that *G. lucidum* possessed antioxidant activities (Mau *et al.*, 2002) and free radical scavenging activities (Baskar *et al.*, 2008).

In addition, polysaccharide fractions from *G. lucidum* were found to accelerate wound healing of acetic acid-induced ulcer in rats (Gao *et al.*, 2004). Polysaccharide krestin (PKS), from *Trametes versicolor* have the mimetic activity of SOD and promoted oxidative

stress relief for cancer bearing hosts (Smith *et al.*, 2004). Significant superoxide and hydroxyl radical scavenging activities have been demonstrated for several mushroom anti-tumour polysaccharides (Smith *et al.*, 2004).

In a study of antioxidant status in delay wound healing type, it was found that low levels of antioxidants accompanied by raised levels of markers, free radical damage play a significant role in chronic wound healing (Rashid & Shukla, 2001). In the search for sources of natural antioxidants and compounds with free radical scavenging activity, several difference species of macrofungi were widely studies on their antioxidants and free radicals scavenging purposes (Chang *et al.*, 2007). *G. lucidum* were discovered to have the ability of removing and reducing the production of oxygen free radicals (Baskar *et al.*, 2008). Since numerous studies demonstrated that oxidative stress mediated by hyperglycemia induced generation of free radicals and it contributes to the development and advancement of diabetes and related complication. Ameliorating oxidative stress through treatment with antioxidants might be an effective strategy for reducing diabetic complications (Ceriello, 2003), in particular the impaired wound healing. Our result showed that application of *G. lucidum* extract incorporated cream may act as exogenous antioxidant cream and free radicals scavenger, in which enhanced wound healing in diabetic rats. The prominent antioxidant properties in medicinal mushroom, *G. lucidum* might be reasonable to play its role in chronic wound healing of diabetic patient.

Histological studies showed that healing impairment is characterized by delayed cellular infiltration and granulation tissue formation, reduced angiogenesis and collagen, and its organization (Coleman, 1982). The mechanism of this alteration is thought to result from production of high level ROS and increased level of apoptosis related to diabetes mellitus, which in turn impairs keratinocyte endothelial cells, fibroblast, and collagen

metabolism (Silhi, 1988). A series of multiple mechanisms including decreased cell, diminished peripheral blood flow, maturation factor response and decreased angiogenesis, all of which can contribute to chronic wound healing in persons with diabetes mellitus (Brem & Tomic-Canic, 2007). In normal conditions, the generation of free radicals is counter balanced by the presence of adequate endogenous antioxidant defences. In diabetics wound healing model, our results showed that wound treated with aqueous cream containing 10% of *G. lucidum* extract has reduced inflammatory cells, more collagen matrix or fibril, more capillary vessels and fibroblast cells proliferation. It appears that antioxidant activity as well as immune modulation of polysaccharides may bring about the wound healing effects of this medicinal mushroom.

It was reasonably that the bioactive compounds of *G. lucidum* contributed synergistically in enhancing wound healing through the mechanisms of antioxidant capacity in diabetic wound healing. It also revealed that the *in vitro* antioxidant activity of extract of *G. lucidum* works under *in vivo* conditions (Shimoi *et al.*, 1996). However, the contribution of antioxidant activities and the underline mechanisms and the defence pathway involved need to be further investigated.

5.5 Conclusions

This study showed that topical application of aqueous cream containing 10% aqueous extract of *G. lucidum* significantly ($p < 0.05$) enhanced wound healing in streptozotocin-induced diabetic rats. The present *in vivo* study showed that the mushroom extract increased the total antioxidant capacity in rats via external application of cream at the wounds site. Aqueous cream containing 10% extract of *G. lucidum* possessed a better wound healing effect than commercial Intrasite gel in diabetic wound healing. Oxidative

stress indices, AOPP and LHP in the diabetic rats were probably due to ROS generated by STZ-induction and the progression of untreated diabetes in experimental rats. Identification of active ingredients in the aqueous extract of *G. lucidum* is warranted. Wound healing activity exhibited by aqueous extract of *G. lucidum* could be due to the synergistic reaction of bioactive compounds; the polysaccharides, triterpenes or adenosine and need to be further elucidated. Since *G. lucidum* is ubiquitous and abundantly grown, it could be an economical therapeutic agent for wound management or a healing promoter, as well as to control abnormal healing. From the perspective in the laboratory experiment and the traditional uses of *G. lucidum* powder in wound management, there is a compelling need to develop an alternative medicine for impaired wound healing, which may help to eliminate amputation and improve quality of life in patient with diabetes.

6. SUGGESTIONS FOR FURTHER STUDY

During the healing process, various growth factors are secreted to accelerate wound healing (Singer *et al.*, 1999). Cytokines and inflammatory mediators released at the wound site are thought to have a key role in regulating healing processes. The important of gene expression and cytokine released when extract of *G. lucidum* was applied need to be further elucidated. Vascular endothelial growth factor (VEGF) is one of the most potent known angiogenic cytokines and promotes all steps in the cascade process of angiogenesis (Ferrara *et al.*, 1997). The observation that certain growth factors have a role in tissues repair has in some cases been further investigated as a candidate therapy in enhancing wound repair. Deficient tissue repair in diabetic and chronic ulcers has been manipulated using platelet derived growth factor (PDGF) and transforming growth factor (TGF) (Davidson *et al.*, 1997). The precise molecular and cellular mechanisms involved in wound healing by extract of *G. lucidum* remain largely unknown. Furthermore, decreased chemotaxis and phagocytosis (Marhoffer *et al.*, 1992), and a reduction in the levels of growth factors (Beer *et al.*, 1997), and the inhibition of fibroblast proliferation have all been suggested to contribute to the observed impairment in wound healing of diabetic rats (Hehenberg *et al.*, 1998). However, the stimulation of growth factors by the extract of *G. lucidum* need to be further investigated.

In the study of pharmatokinetic and pharmacodynamics of pure compounds in traditional herbs are thought to be involved in the activity of a traditional medicine. The crude extract of a medicinal mushroom, however, may contain compounds that are not

active individually but affect the pharmacological parameters of the pure compounds, e.g. in intake, metabolism and excretion or by synergistic or antagonistic effects in a biological system should be considered. The suggestion that crude extract of *G. lucidum* with the standardization of active ingredients could be used in these studies.