EVALUATION OF WOUND HEALING POTENTIAL, ANTIOXIDANT ACTIVITY, ACUTE TOXICITY AND GASTRO-PROTECTIVE EFFECT OF 2-PENTADECANONE IN ETHANOL INDUCED GASTRIC MUCOSAL ULCERATION IN RATS

SAREH KAMRAN

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SAREH KAMRAN

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EVALUATION OF WOUND HEALING POTENTIAL, ANTIOXIDANT ACTIVITY, ACUTE TOXICITY AND GASTRO-PROTECTIVE EFFECT OF 2-PENTADECANONE IN ETHANOL INDUCED GASTRIC MUCOSAL ULCERATION IN RATS

ABSTRACT

Wound is an injury to normal tissue that can happen either externally or internally such as wound in skin tissue or stomach tissue. Compound 2-pentadecanone a ketone derivative, has been identified in some plants, and was reported to have anti-bacterial, antioxidant and anti-inflammatory activities. The aim of this study was to evaluate skin wound healing and gastro-protective potential effect of 2-pentadecanone in *Sprague dawley* rats. Acute toxicity assay was performed to detect safety of 2-pentadecanone. In the evaluation of skin wound healing, dorsal neck of rats were induced wounds and topically treated with 2-pentadecanone for 10 days. Meanwhile, for gastroprotective study, rats were pre-treated orally with 2-pentadecanone and gastric ulcer was induced by absolute ethanol. Next, skin tissues and stomach tissues were collected for tissue homogenate preparation. Analysis of anti-oxidant effect of 2-pentadecanone in tissue homogenate (superoxide dismutase, catalase and malonialdehyde level determination), percentage of skin wound closure, percentage of stomach ulcer inhibition, macroscopic observations, histological staining (Hematoxylin and Eosin, Masson Trichrome and periodic acid schiff) and immunohistochemistry (HSP70 and BAX) were performed on skin and stomach tissues. Additionally, the level of nitric oxide was measured in stomach tissue. Anti-oxidant effect of 2-pentadecanone on both skin and stomach tissue homogenate was detected. Treatment of skin wounds and pre-treatment of stomachs with 2-pentadecanone resulted in over expression of superoxide dismutase and catalase enzymes and reduction in malonialdehyde level in tissue homogenate in both samples. In studies, percentage of wound closure, percentage of stomach ulcer inhibition,

macroscopic and histological analysis of wounded skin and stomach tissues revealed a positive result of 2-pentadecone applications. Immunohistochemistry assay showed up-regulation of Hsp70 and down-regulation of Bax protein in skin granulation tissue of treated groups with 2-pentadecanone and stomach tissues of pre-treated rats with this compound. The present results suggested that 2-pentadecanone has promising wound healing and gastro-protective potential effect.

Key words: wound healing, gastric ulcer, 2-pentadecanone, antioxidant, HSP70/Bax

PENILAIAN POTENSI PENYEMBUHAN IKUA, AKTIVITI ANTIOKSIDAN, KETOKSIKAN AKUT DAN KESAN PERLINDUNGAN-GASTRO 2-PENTADECANONE DALAM ETANOL MENYEBABKAN GASTRIK MUKOSA ULSER PADA TIKUS

ABSTRAK

Luka adalah gangguan kepada struktur normal pada permukaan kulit dan juga permukaan perut. Derivatif ketone seperti 2-pentadecanone yang ditemui di dalam tumbuhan telah dilaporkan mempunyai aktivito-aktiviti seperti, anti-bakteria, anti-oksida dan anti-inflamasi. Justeru itu kajian ini, bertujuan untuk mengkaji potensi kesan 2-pentadecanone dalam penyembuhan luka kulit dan perlindungan-gastro bagi tikus Sprague Dawley (SD). Dalam kajian ini, ujian ketoksikan akut dijalankan bagi penilaian keselamatan 2-pentadecanone. Bagi kajian potensi penyembuhan luka kulit, pemotongan luka luar kulit dibuat di bahagian belakang leher dan dirawat selama 10 hari. Manakala bagi potensi perlindungan-gastro, tikus SD dirawat dengan 2-pentadecanone secara oral dan kemudiannya dilukakan permukaan perut dengan etanol mutlak bagi menghasilkan ulser gastrik. Kemudian, tisu kulit dan tisu perut dikumpul bagi penyediaan tisu homogenate. Analisis dan ukuran seperti kesan antioksidan 2-pentadecanone (penentuan superoxide dismutase (SOD), catalase (CAT) dan malonialdehyde (MDA)) pada homogenat tisu; peratusan penutupan luka kulit, peratusan perencatan ulcer, makroskopik, histologi serta immunohistokimia (IHC) (HSP70 dan Bax) bagi tisu kulit dan tisu gastrik/perut. Tambahan bagi tisu gastrik/perut, aras nitrik oksida juga diukur. Kesan antioksidan 2-pentadecanone dikesan dalam kedua-dua kulit dan tisu perut. Rawatan luka kulit dan pra-rawatan perut dengan 2-pentadecanone telah menghasilkan lebihan ekspresi SOD dan enzim katalase dan pengurangan aras MDA di dalam tisu homogenat kedua-dua tisu. Dalam kedua-dua kajian, analisis peratusan penutupan luka kulit, peratusan perencatan ulser, makroskopik

dan histologi bagi tisu yang cedera dan tisu gastrik menunjukkan kesan positif 2-pentadecanone. Analisis IHC mengesahkan kenaikan regulasi protein HSP70 dan penurunan regulasi protein Bax dalam tisu granulasi dan tisu perut kumpulan yang menerima 2-pentadecanone. Kesimpulannya, 2-pentadecanone telah menunjukkan kesan positif pada proses penyembuhan luka dan kesan perencatan ulser pada tikus SD.

Kata kunci: penyembuhan luka, ulser gastrik, 2-pentadecanone, antioksida, Hsp70/Bax

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LIST OF SYMBOLS AND ABBREVIATIONS

AEU	:	Animal Experimental Unit
A0	:	Initial wound area
At	:	Final wound area
BHB	:	B-hydroxybutyrate
CAT	:	Catalase
CMC	:	Carboxymethyl cellulose
CYP450	:	Cytochrome P450 monooxygenases
CO_2	:	Carbon dioxide
CF	:	Collagen fiber
D	:	Dermis
DILI	:	Drug induced liver injury
DAB	:	Diaminobenzidine tetrahydrochloride
E	:	Epidermis
ECM	:	Extra cellular matrix
FDA	:	Food and Drug Administration
GT	:	Granulation tissue
GST	:	Glutathione-s-transferase
GR	:	Glutathione reductase
GPX	:	Glutathione peroxidase
GA	:	Glycogen accumulation
HCL	:	Hydrochloric acid
Hsp70	:	Heat shock protein 70
H & E	:	Hematoxylin and Eosin
H_2O_2	:	Hydrogen peroxide
Hr	:	Hemorrhagic bands

I %	:	Inhibition percentage
IN	:	Inflammatory infiltration
MDA	:	Malonialdehyde
MT	:	Masson Trichrome
Nrf2	:	Nuclear factor erythroid derived 2-related factor 2
NF-kB	:	Nuclear factor kappa light chain enhancer of activated B cells
NLRP3	:	Nucleotide binding domain, leucine rich containing family and pyrin domain containing-3
NSAID	:	Anti-inflammatory drugs
NF-$_K$B	:	Nuclear factor kappa B
NO	:	Nitric oxide
NLRs	:	Nucleotide bindings and oligomerization domain like receptors
OECD	:	Organization for Economic Co-operation and Development
O^-_2	:	Superoxide anion
OH^-	:	Hydroxyl radicals
PBS	:	Phosphate buffered saline
PPIs	:	Proton pump inhibitors
ROS	:	Reactive oxygen spices
RBC	:	Red blood cells
SOD	:	Superoxide dismutase
S	:	Scar
SD	:	*Sprague Dawley*
TBA	:	Thiobarbituric acid
TNF-α	:	Tumor necrosis factor α
T.protein	:	Total protein
TGF- β1	:	Transforming growth factor beta 1

UV	:	Ultra violet
VEGF	:	Vascular endothelial growth factor
WHO	:	World Health Organization

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 General introduction

From the very beginning of life, human begins were totally dependent on nature for the purpose of shelters, cloth, food and medicine production and countless of other basic requirements. Although, not every claim on the therapeutic effect of medicinal plants found to be proven scientifically, treatment based on natural products is rooted from the empirical findings of centuries. The first records of medicinal plants were written on clay tablets in cuneiform belongs to Mesopotamia from 2600 years ago, which has claimed that the oil of some medicinal plants could fight against various illnesses ranging from colds to inflammation and infections. Natural products have drowned attention in medicine field for various purposes such as chemotherapy (Gurib-Fakim, 2006), wound healing (Boakye *et al*., 2018) and inhibition of gastric ulcer (Hajrezaie *et al*., 2015).

Medicinal plants are highly considered in pharmacological assessments due to the presence of phytochemicals. Generally, phytochemicals are natural and biologically active compounds produced by plants to protect plants against critical environmental conditions.

In general, pharmaceutical industry can apply its techniques in terms of drug developments, using naturally occurring phytochemicals. The possibility to obtain phytochemicals alternatively by chemical synthesis rather than extraction from their original sources offers a sustainable production strategy in drug development industry (Zerazion *et al*., 2016).

2-pentadecanone, which is a naturally occurring bioactive compound can be also synthesized. This compound belongs to the family of ketones. Ketone molecules were reported to exert pharmacological activities such as fighting against oxidative damage and inflammation (Walia *et al*., 2012; Gunalan *et al*., 2016; Baratelli *et al*., 2012; Taveira *et al*., 2003; Goh *et al*., 2012; Haces *et al*., 2008; Dugasani *et al*., 2010; Korting *et al*., 1993).

Skin wound is described as disruption of the cellular, anatomical and functional integrity of the skin structure. Wound healing process is known as a complex mechanism that involves overlapping phases that function to regain the normal structure of the skin tissue (Lin *et al*., 2012). There are four main phases involved in wound healing process: 1: hemostasis, 2: inflammation, 3: proliferation and 4: remodeling that work to overcome skin structure disruption (Ho, 2013; Soliman *et al*., 2018).

Gastric ulcer is a widely occur gastrointestinal disorder that appears as lesions in the gastrointestinal tract. About 14.5 million individuals were reported to be affected by gastric ulcer worldwide (Chen *et al*., 2015). Progressed gastric ulcer was reported to be associated with short term mortality in 30% of patients (Ghosh *et al*., 2018). In 2019 it was reported that this chronic disease has affected up to 10% of world population (Kuna *et al*., 2019). Factors such as consumption of alcohol, being under stress, prolong consumption of non-steroidal anti-inflammatory drugs (NSAIDs); poor diet and smoking are examples that lead into the development of gastric ulcer by disrupting the normal balance between defensive and offensive factors in mucosal barrier. Nowadays, great achievements such as anti-acids drugs or proton pump inhibitors are being used against gastric ulcers, however the side effects and the high cost lead into the search for new therapeutic agents (Chen *et al*., 2015; Kuna *et al*., 2019).

1.2 Research significance and justification

The records have shown that 1 to 2% of the population in developed countries experience chronic wounds during their life time (Sen *et al*., 2009). Although, there are available drugs used to recover the wounded tissue, search for a new therapeutic agent is still ongoing. This is to achieve more effective and enhanced drug in comparison to the available drugs in the market. The significance of this study emphasizes on the safety, skin wound healing and gastro-protective effect of 2-pentadecanone as a natural and pure compound. These discoveries introduce 2-pentadecanone as a new potential therapeutic agent comparable to the standard drugs (omeprazole and intrasite gel) drugs in the market.

1.3 Problem statement

Little is known on pharmacological effect of 2-pentadecanone, however it was detected as a minor and major compound in some medicinal plants, which the crude extract showed some pharmacological activities (Walia *et al*., 2012; Gunalan *et al*., 2016; Baratelli *et al*., 2012; Taveira *et al*., 2003).

Wound recurrence is a significant issue that emerge in 40% of cases (Rosenblum *et al*., 2017). Indeed, non-healing wounds appear in a large fraction of the world population, estimated 1 to 2% of the population in developed countries (Sen *et al*., 2010). This health issue has caused major problems to patient and health care system, for example slow healing tendency of diabetic wounds (Han & Ceilley, 2017).

In the case of stomach ulcer, 40% to 80% reoccurrence of this disease is detected after therapy is stopped (Abood *et al*., 2014). One consideration is that, long-term consumption of anti-ulcer drugs bring up side effects such as arrhythmia and anemia in

most cases, therefore search for a more advanced, and efficient antiulcer drug is important (Abood *et al*., 2014).

1.4 Aim and Objectives

Subsequent the problem statement, in general, the aim of this study is to evaluate skin wound healing and gastro-protective potential effect of 2-pentadecanone in *Sprague dawley* rats. The following are the objectives of this study:

- To evaluate acute toxicity effect of 2-pentadecanone
- To evaluate antioxidant activity of 2-pentadecanone in both skin and gastric tissue homogenate
- To evaluate skin wound healing effect of 2-pentadecanone in *SD* rats
- To evaluate gastric ulcer inhibitory effect of 2-pentadecanone in ethanol induced gastric ulcer in *SD* rats

CHAPTER 2: LITERATURE REVIEW

2.1 Medicinal plants and their pharmacological activities

2.1.1 Natural medicine

Plants are ancient natural sources that have the potential of synthesizing medicinal compounds. The characterization of these compounds has offered the discovery of new, inexpensive and highly effective drugs. It was reported that 80% of the world's population relies on the natural medicinal sources for their primary health care (Nafiu *et al*., 2017). The importance of natural products was reported, as United States Food and Drug Administration (FDA) approved several new drugs inspired by natural products in 2019 (Beatriz & Albericio, 2019).

2.1.2 Phytochemicals

Natural product's bioactivity is the result of phytochemicals that produced by plants to protect them against risk factors such as herbivore or pathogens. Aldehyde, alcohols, amides, ketones, carboxylic acids and other phytochemicals as well were obtained as a result of phytochemical screening from some medicinal plants (Ahmad *et al*., 2014). These phytochemicals have the potential to defend human beings against pathogens. Some phytochemicals are known to exert therapeutic effects such as antioxidant properties and inhibitory effect against carcinogens, they also have the potential to provide nutrition for normal cell health and repair (Njerua *et al*., 2013). In relation to this research, medicinal plants have a recent record of being used in wound healing study (Lordani *et al*., 2018) and gastric ulcer prevention (Kuna *et al*., 2019). Phytochemicals promote their promising effect by presenting antioxidant activity, stimulating immune mechanism, modulating hormone metabolism, exerting anti-bacterial and anti-viral effect or by binding to specific sites on cell membrane to block interaction of pathogens with cell wall (Njerua *et al*., 2013). They may also leave a

positive effect by exerting anti-inflammatory response (Oguntibeju, 2018) and through the regulation of protein expression (Ajit *et al*., 2016). Moreover, previous studies have suggested establishment of wound dressings from medicinal plants. Large number of plants and their active compounds with significant antioxidant and anti-inflammatory activities were reported as ideal agents for wound treatment (Georgescu *et al*., 2016).

2.1.3 2-pentadecanone

In 2015, the plant *Labisia Pumila* was screened by Gas Chromatography Mass Spectrometry technique for its bioactive compounds. As a result, 2-pentadecanone was identified as a major compound in this plant (Kamran, 2015). Moreover, it has been reported that this compound was identified in other plants (Table 1.1) with wound healing, antioxidant and anti-inflammatory activities (Walia *et al*., 2012; Gunalan *et al*., 2016; Baratelli *et al*., 2012; Taveira *et al*., 2003). 2-pentadecanone belongs to the family of ketone. It is well known that ketones were recommended as a cure for epilepsy disorder. The principal pharmacological activity of ketones is due to its antioxidant effect, which improves mitochondrial function followed by limiting oxidative stress. Ketones exert their effect by exciting the cellular endogenic antioxidant process with the activation of nuclear factor erythroid derived 2-related factor 2 (Nrf2), which regulates expression of antioxidant proteins. Furthermore, ketones were suggested to induce anti-inflammatory activity due to their suppressive potential on nuclear factor kappa light chain enhancer of activated B cells (NF-kB) and nucleotide binding domain, leucine rich containing family and pyrin domain containing-3 (NLRP3) inflamosome (Pinto *et al*., 2018). In addition, ketone supplementation was reported to enhance wound healing process by enhancing proliferation, decreasing reactive oxygen spices (ROS) generation and resolving inflammation (Kesl *et al*., 2014). Moreover, some chemical compounds that contain ketone functional group have shown antiulcer

properties (Siqueira *et al*., 2011). With regards to 2-pentadecanone, a study published in 2019, mentioned the anti-inflammatory effect of this compound, which identified from *Marantodes Pumilum* (Siyumbwa *et al*., 2019).

Table 1.1: Plants that contain 2-pentadecanone and their pharmacological effect.

Activity	Plant name
Wound healing	*Bauhinia variegata*
Antioxidant	*Malus domestica* (Walia *et al*., 2012) *Bauhinia variegata* (Gunalan *et al*. 2016) *Terminalia catappa* (Baratelli et al., 2012)
Anti-inflammatory	*Malus domestica* (Taveira *et al*., 2003) *Bauhinia variegata* (Gunalan *et al*. 2016) *Terminalia catappa* (Baratelli et al., 2012)

Furthermore, Plants such as *Peganum harmala* and *Eclipta alba*, which contain 2-pentadecanone analog (6,10,14-trimethyl-2-pentadecanone) were reported to improve wound healing activity. Also, *Eclipta alba* has been reported to exert antioxidant activity (Moussa & Almaghrabi, 2016). Previously, 2-pentadecanone was found among the components of *Bauhinia variegata*, a plant with anti-cancer, anti-viral, anti-bacterial, anti-diabetic and anti-inflammatory activities (Gunalan *et al*., 2016). In regards to support the current study, it was suggested that compounds with antioxidant and anti-inflammatory properties may improve wound healing process (Kant *et al*., 2014). Therefore, in the present study 2-pentadecanone was tested against external and internal wounds.

Malus domestica, which contain 2-pentadecanone compound has showed antioxidant, anti-inflammatory and anti-microbial activities (Walia et al., 2012b). In addition, 2-pentadecanone was also found among the phytochemicals of fraction I in *Bauhinia variegata* plant. This plant was tested for its anti-inflammatory effect where all the phytochemicals including 2-pentadecanone showed anti-viral, anti-bacterial, anti-diabetic and anti-inflammatory effect (Gunalan *et al*., 2014). Moreover, in a study

where *Bauhinia variegata* was evaluated for its anticancer activity, 2-pentadecanone was detected in the active fraction of the plant (Gunalan *et al*., 2016).

In theory 2-pentadecanone can enhance the process of wound healing and inhibit gastric ulcer formation by blocking free radicals and prevent inflammation. The chemical structure of this compound is represented in figure 2.1.

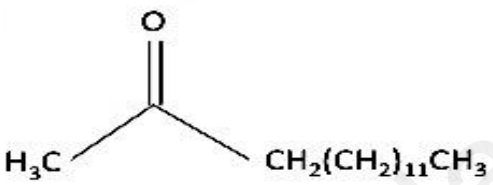

Figure 2.1: Chemical structure of 2-pentadecanone ($C_{15}H_{30}O$). Molecular weight: 226.40 (Adapted from www.sigmaaldrich.com).

2.1.4 Antioxidants

Antioxidants are substances that are either synthesized or being found in natural sources such as fruits and vegetables, and their significant role is the ability to inhibit or postpone cell damage. Plants and animals maintain a complex system of antioxidants such as vitamin C, vitamin A and vitamin E, as well as enzymes such as superoxide dismutase (SOD) and catalase (CAT). Traditionally, herbal medicine were popular sources of antioxidants that have protected people from the harmful effect of free radicals, and nowadays they are being used in dietary supplements and also to prevent certain diseases such as cancer and heart disease (Yadav *et al*., 2016). With regard to wound healing and gastro-protective studies, antioxidant enzymes such as SOD and CAT revealed their potential effect to induce wound healing mechanism (Abood *et al*., 2015) and prevent gastric ulcer formation (AL-Wajeeh *et al*., 2017).

2.1.5 Protective effect of antioxidants against free radicals

Free radicals are generated as a result of normal cellular metabolism. These

molecules are highly reactive and once they are produced, they abstract electrons from other molecules to gain stability. As a result, more free radicals are generated over the loss of electrons in the attacked molecules, which produce an electron chain reaction that is harmful to cells (Phaniendra *et al*., 2015).

Oxidative stress is an imbalance status between the free radicals and antioxidants, which leads to the incidence of several health complications. The protective advantage of antioxidants against free radicals was highlighted in several studies. Certain antioxidant enzymes such as SOD and CAT have been asserted for their healing and inhibitory effect against several illnesses (Ighodaro & Akinloye, 2017).

Antioxidants leave their impact on living cells based on four defensive lines. The first defensive line of antioxidant molecules acts to inhibit the generation of free radicals by neutralizing potential free radical molecules. SOD and CAT are of the main antioxidant enzymes of first defensive line. SOD exerts its defensive effect by catalyzing the dismutation of the superoxide radicals and produce hydrogen peroxide. The damaging effect of the reactive molecule, hydrogen peroxide is eliminated by the action of CAT enzyme. This enzyme breakdown hydrogen peroxide into harmless molecules (Ighodaro & Akinloye, 2017; Szuster-Ciesielska *et al*., 2004). Second defensive line of antioxidants protect cells by scavenging free radicals to block electron chain initiation. They donate electrons to free radicals to increase their stability and in such case, they reduce the damaging effect of free radicals. Third defensive line of antioxidants plays its role after free radicals have damaged cells. Those antioxidants repair enzymes that restore the damaged DNA, proteins and lipids, and also they prevent over accumulation of toxic substances resulted from free radical activities. The popular examples are the DNA repair enzyme system and proteolytic enzymes that are

located in cytosol and mitochondria of cells. The fourth defensive line uses the signals required for the generation of free radicals to produce appropriate antioxidant at the right side to block the action of reactive molecules (Ighodaro & Akinloye, 2017).

Lipid peroxidation is a process in which free radicals or ROS attack lipid molecules. This process results in oxidative damage, mainly organ or tissue injury. One of the most toxic products of lipid peroxidation process is malondialdehyde (MDA), and it has been widely used as a biomarker of lipid peroxidation. The most common test to detect MDA is thiobarbituric acid (TBA) test, which act based on the high reactivity of TBA and MDA toward each other (Ayala *et al*., 2014).

2.1.6 Inhibitory effect of ketones on NLRP3 inflammasome

NLRP3 protein belongs to nucleotide bindings and oligomerization domain like receptors (NLRs) family, which include pyrin domain containing protein 3 (Shao *et al*., 2015). The NLRP3 inflammasome is activated as a response to signals such as tissue damage and it controls secretion of pro-inflammatory mediators like IL-1β and IL-18 (Leemans *et al*., 2011). A study has established inhibitory effect of ketone metabolites on the production of pro-inflammatory mediators released by NLRP3 inflammasome. B-hydroxybutyrate (BHB) is a ketone metabolite that showed suppressive effect on NLRP3 inflammasome, as a result the ketone metabolite was reported as a potential agent to diminish inflammation (Shao *et al*., 2015).

2.2 Skin tissue injury and wound healing

2.2.1 Anatomy of skin

The largest body's organ that covers the entire external surface of body is skin. Epidermis, dermis and hypodermis are the main layers of skin that have different anatomy (Yousef & Sharma, 2018). Anatomy of the skin is shown in Figure 2.2.

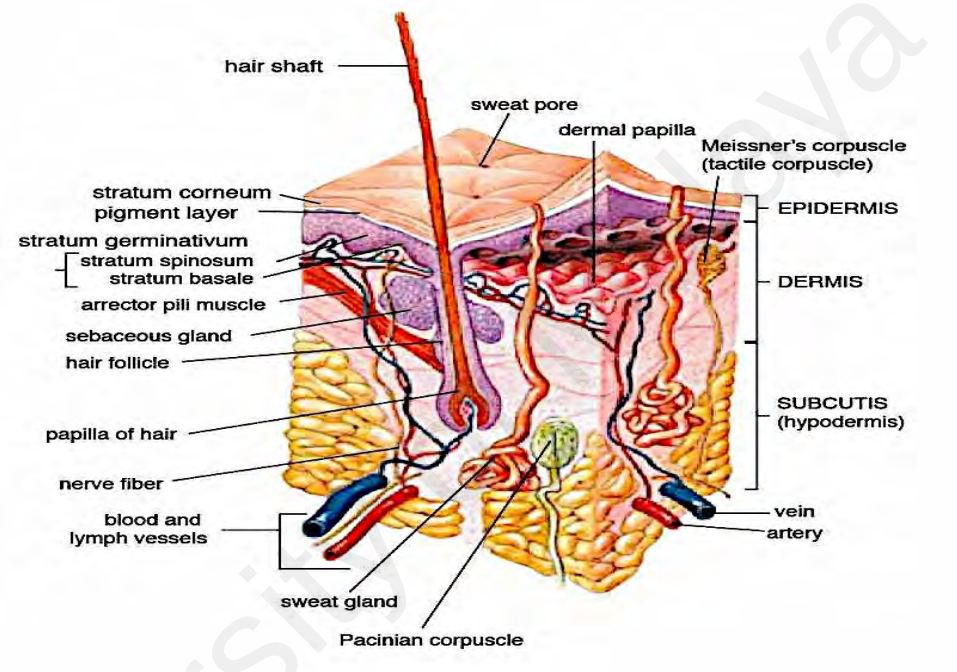

Figure 2.2: Anatomy of the skin (Yousef & Sharma, 2018).

2.2.2 Wound definition and classification

Skin wound refers to an injury in the skin tissue due to pierce, cut, or a break that could be exerted by physical, chemical, microbial or immunological disruption that ruin the normal integrity of the tissue structure. This can damage either epithelium or the underlying tissues as well. Wounds can be the result of blunt trauma, penetrating trauma, surgery, chemical injury, thermal injury, high temperature (burn) and radiation. Significant signs arise upon injury are unusual flushing of skin, swelling, redness and heat at the injured area which is followed by possible fever and infection (Pandey & Arun, 2017).

Wounds are classified into open and closed wounds according to the related wound creation cause (Tiwari, 2012), (Pandey & Arun, 2017), also categorized into acute and chronic wounds based on the physiology of wound healing (Pandey & Arun, 2017). Open wounds are found when blood finds a way to the external surface. Examples of open wounds are incised wounds that raise minimal tissue disruption. These wounds are developed as a result of a sharp object such as knife. Laceration wounds that trigger tissue damage and its detriment. Scratch over a rough surface, which harm epidermis layer of the skin. Puncture elisions that are the results of exposure to puncturing objects such as needle. Penetration wounds, which happen due to penetration of an object such as knife deep into the skin and gunshot injury that is caused by hitting a driving object such as bullet. Closed wounds occur when blood find its way out of the circulatory system but not to the external surface of the skin, which result in appearance of bruises. Examples of closed wounds are contusions wounds that are induced by a blunt force trauma and hurt the underneath skin layers. Another example is the hematomas wounds that are caused by ruptured blood vessels followed by the accumulation of blood beneath the skin and crush injuries, which appear as a result of skin being under an excessive force for extended period (Nagori & Solanki, 2011).

Acute wounds such as cuts or surgical incisions represent skin injuries that respond to wound healing process within the expected healing duration. On the other hand, wounds that do not respond to the healing process and remain unrecovered for more than one month are considered as chronic wounds (Pandey & Arun, 2017; Sen, 2019).

2.2.3 Factors affect wound healing

There are some factors such as dehydration, infection, necrosis and pressure that delay wound healing process. Dehydration of cells results in the formation of a scab at the site of injury and hinder process of healing, while a moist wound area facilitates migration of epidermal cells which in turn induce epithelialization. Infection is evidenced by excretion of exudate, induration, erythema and fever. In addition, slough with a white; moist and loose appearance and scar with a dry, thick and black appearance are signs of necrotic tissue, which delay wound healing. Moreover, the result of excessive and constant pressure on the site of injury affect blood supply to the capillaries, which in turn lower blood circulation to the neighboring area and delay wound healing (Thomas Hess, 2011). Furthermore, age, obesity, chronic disease such as diabetes mellitus, immunosuppression, diet, radiation therapy and vascular insufficiencies are factors that affect wound healing (Guo & DiPietro, 2010; Patel *et al*., 2019; Larouche *et al*., 2018; Brown & Phillips, 2010; Jacobson *et al*., 2017; Anderson & Hamm, 2012; Thomas Hess, 2011).

2.2.4 Wound healing mechanism

Skin is the outermost protective layer that defenses us against the external environment and composed of epidermis, dermis and subcutaneous layer. Upon skin injury, the synergetic connection of several dermal cells at different levels is necessary to induce wound healing process, which involves four overlapping phases. Hemostasis, the first phase of wound healing mechanism, by the aid of platelets accumulation and coagulation factors, blocks the loss of blood. The second phase of this process is inflammatory phase, which induce the release of cytokines, growth factors and inflammatory cells at the site of injury to fight against pathogens. The third healing phase is proliferation, where retrieval of extracellular matrix (ECM) takes place. This

phase triggers certain processes, in specific, re-epithelialization, angiogenesis and granulation tissue formation. Remodeling, the last healing phase involves maturation of scar tissue (Soliman *et al*., 2018).

2.2.5 Wound healing phases

Wound healing process includes four overlapping phases. These are hemostasis, Inflammatory, proliferation and remodeling phases (Soliman *et al*., 2018). Wound healing process and the events occur during each phase are shown in Figure 2.3.

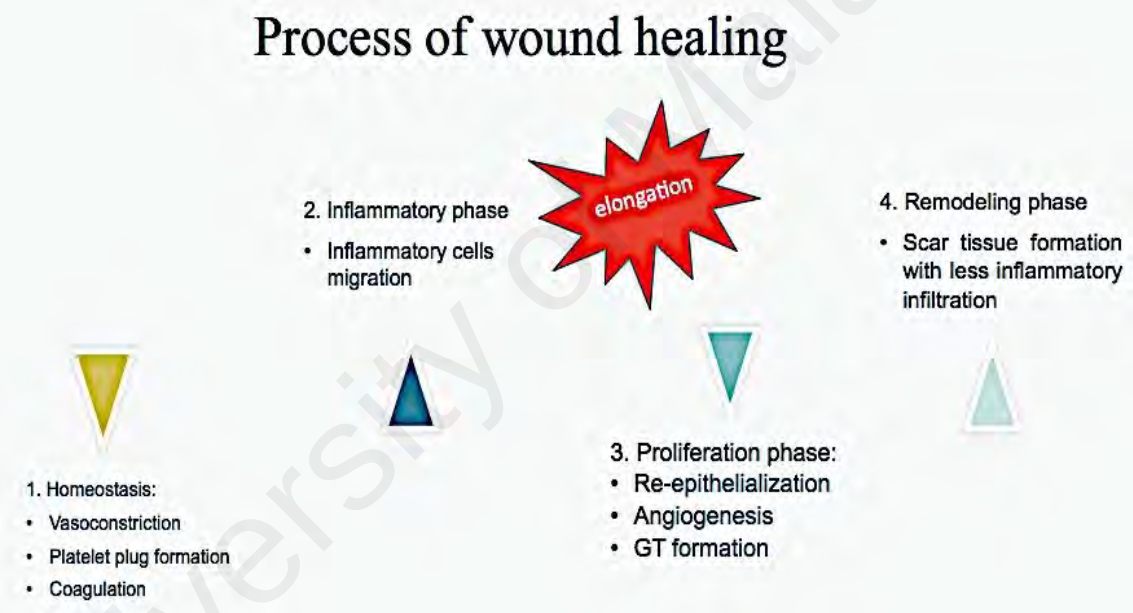

Figure 2.3: Process of skin wound healing (Soliman *et al*., 2018).

2.2.6 Wound care dressings

Development of chronic wounds and morbidity can be raised as a result of defective wound management. The main consideration in wound care is the removal of debridements, or in another term non-viable tissue substances. This concept is achievable, either by surgery or autolytic/enzymatic mechanism. In both cases, the main objective is to expose healthy tissue that is capable of proliferation and epithelial cell migration to the wound bed. The result of this concept, keep away necrotic

debridements that support infection at wound site. Application of surgical debridement is of limited use, as timing and frequency of surgery is extremely variable depending on the type of wounds. However, this technique is a significant element of wounds management. On the other hand, autolytic debridement is defined as the self-activation of endogenous enzymes associated with fibrin degradation, and it is most likely to happen in a hydrated wound environment. The hydration of wound environment is achievable through the application of some wound dressings (Han & Ceilley, 2017). High variety of wound dressings are developed to protect wounded tissue from infection and support wound healing process. Dressing selection is directly correlated to several factors such as type, depth, location and extent of wound (Rezvani Ghomi *et al*., 2019). Dressings with miniaturization property reinforce wound healing mechanism by activating factors such as hypoxia-inducible factor-1, which regulates oxygen homeostasis within the wound environment (Han & Ceilley, 2017) and enhance re-epithelialization (Jones *et al*., 2006).

2.2.7 Intrasite gel, a standard wound dressing

Intrasite gel has a colorless transparent appearance. Its composition includes 2.3% of carboxymethyl cellulose (CMC) and 20% of propylene glycol as humectant. It has the ability to absorb extra exudate and induce hydration at the wound site with no hurt in the tissue. Intrasite gel supports autolytic debridement mechanism by inducing hydration, the moist environment provided by intrasite gel facilitates re-epithelialization process. Despite the exudate absorbent ability of intrasite gel, it is not suitable to be applied on extremely exudative wounds (Smith and Nephew Healthcare Limited).

2.2.8 CMC, a negative control used in wound healing study

CMC is a thickening and gelling agent. It is a water soluble, odorless and tasteless polymer that is defined as cellulose gum (Arancibia, Navarro-Lisboa, Zúñiga, & Matiacevich, 2016). Several studies have used CMC as a negative control in wound healing studies (Dhiyaaldeen *et al*., 2014; Moghadamtousi *et al*., 2015).

2.3 Drug metabolism and acute toxicity

The scientific and ethical considerations suggest to detect the safety of any potential drug before its administration into human body (Sewell *et al*., 2016). Unanticipated drug toxicity is a key factor for drugs withdrawal from the pharmaceutical market. The two major toxic effects of drugs are hepatotoxicity and nephrotoxicity that discourage considering some medications. These drugs fail on the basis of safety in animals and their translation to human safety (Anadón *et al*., 2014). Liver is a vital organ for survival; it takes responsibility of metabolizing chemical drugs. Hence, it is susceptible to drug toxicity and induced injury (Gu & Manautou, 2012). Kidneys also are highly exposed to drug toxicity. These organs have a crucial role in the renal arterial blood flow, glomerular filtration, tubular fluid formation and exit of urine into the urogenital system. Drug toxicity affect renal function by interfering any of these steps (Pazhayattil & Shirali, 2014). Therefore, acute toxicity assay is usually performed by administration of a single dose of a potential compound in animals such as rats. The liver and kidneys of the examined animals are assessed to determine the safety of the tested drug (Loha *et al*., 2019).

2.4 Stomach tissue

2.4.1 Anatomy of stomach

Stomach is characterized as the most significant organ of digestive system and links

esophagus to small intestine. It is defined as a muscular biological structure with capacity to digest food. Cardia, fundus, body and pylorus are the major portions of stomach (Chaudhry & Peterson, 2019). Figure 2.4 represents the main sections of stomach.

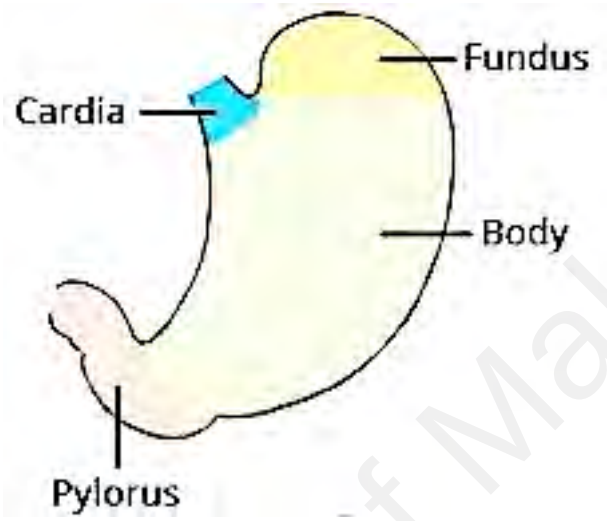

Figure 2.4: Anatomy of stomach. (Adapted from Teach Me Anatomy, https://teachmeanatomy.info/abdomen/gi-tract/stomach/).

Histologically, stomach wall is constructed of four main layers, mucosa, submucosa, muscularis externa and serosa (Chaudhry & Peterson, 2019). Figure 2.5 illustrates layers of stomach wall.

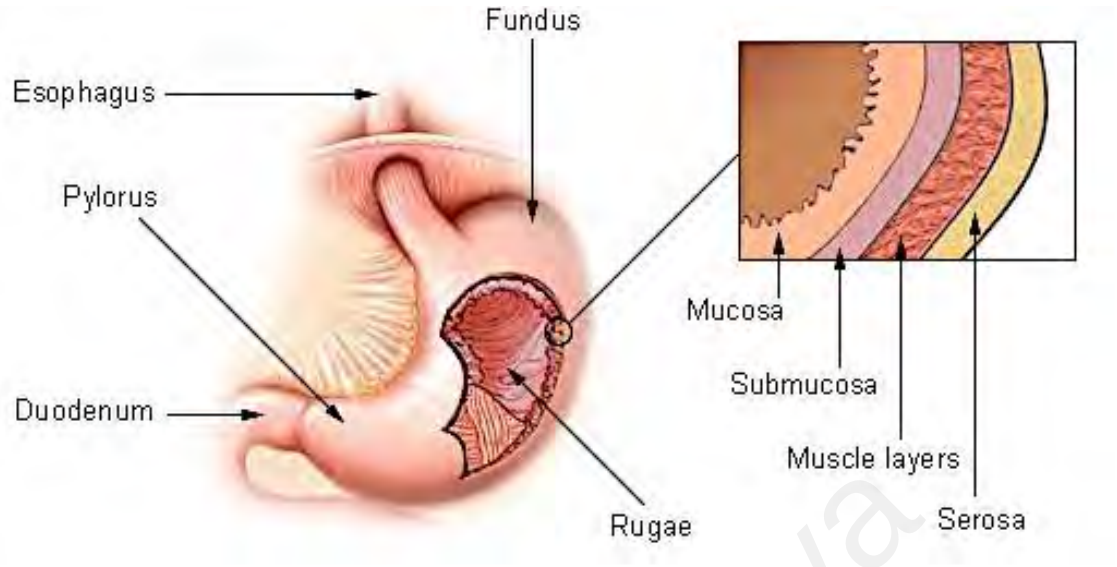

Figure 2.5: Layers of stomach wall. (Adapted from Lumencandela, https://courses.lumenlearning.com/boundless-ap/chapter/layers-of-the-alimentary-canal/).

Mucosa is the innermost layer and is covered by epithelial tissue and contains gastric glands to release gastric juice. To have a more specific explanation, fundus in particular, secret gastric juice and cardia is responsible for the secretion of mucus that covers the deepest section of mucosal wall. Secretion of mucus is necessary to mask stomach muscles and defend it against digestion by gastric juice. Submucosal layer is consisted of packed connective and accommodate blood vessels, lymphatic vessels and nerves. Submucosal layer contains folding known as rugae, to allow contractions and facilitate food passage into the stomach. Muscularis externa is consisted of an inner oblique layer that is specific to stomach, and function to churn up foods during mechanical digestion. Oblique layer is followed by a middle circular layer that become thicker in the area of pylorus to develop pyloric sphincter, and aimed to control the output of stomach into duodenum. Serosa, the outermost layer of stomach wall is constituted of multiple connective tissue layers and links the peritoneum (Chaudhry & Peterson, 2019).

2.4.2 Gastric ulcer

Ulcer in gastric region is characterized by acidic environment, which leads to mucosal destruction on endothelial wall followed by emergence of pain and discomfort. Under normal physiological condition the acidity level of the stomach is 0.5 to 1, an increase in the acidity level damages mucosal layer. This results in the development of sores in various gastric locations such as the lining of esophagus (swallowing pipe), stomach or duodenum (first section of small intestine). Peptic ulcer disease highlights painful ulcers either in the lining of the stomach or duodenum. Development of ulcer in the stomach is defined as gastric ulcer, while ulcer emergence in the first part of the intestine is defined as duodenum ulcer. Indeed, peptic ulcer includes both gastric and duodenal ulcers, a condition that has threatened a high number of population all over the world with a great mortality rate (Singh *et al*., 2017).

Gastric ulcer is a condition of tissue destruction to the depth of gastric mucosa (Abood *et al*., 2014). This acid related disease is known as illness of the twentieth century. Epidemiological outcomes of this disease have shown the importance of geographical changes in incidence of this illness. It is agreed that gastric ulcer occur as a result of an imbalance between aggressive (gastric acid) and defensive (bicarbonate) factors, supporting destruction of mucosal integrity. Some of the other factors support gastric ulcer formation are NSAIDs, smoking, alcohol consumption (Kumar *et al*., 2019) and *Helicobacter pylori* (*H. Pylori*) (Wroblewski *et al*., 2010; Kalisperati *et al*., 2017).

2.4.3 Complications of gastric ulcer disease

There are complications associated with gastric ulcer disease such as: bleeding, perforation, gastric outlet obstruction and gastric malignancy. Among this list of complications, bleeding occur most frequently in 15 to 20% of cases. Gastrointestinal bleeding is a life threatening condition that requires immediate medical care (Reddy & Marsicano, 2018).

Perforated gastric ulcers are reported in 2 to 14% of all cases, this condition is associated with acute abdomen that highlights the risk of mortality and morbidity. The prevalence rate of perforated gastric ulcer is about 5% in patients experiencing gastric ulcer disease with a reoccurrence rate of 12.2%. Perforated ulcers results in the leakage of gastric juice and gas into the peritoneal cavity and lead to chemical peritonitis, followed by a sudden abdominal pain that requires emergency care (Chung & Shelat, 2017).

Gastric outlet obstruction appears as a result of gastric ulcer disease in less than 5% of patients. It is a condition of obstruction in the distal stomach, pylorus or proximal duodenum as a result of acute edema. Gastric outlet obstruction is a preterminal incident in patients that show advanced stomach malignancies. In this condition surgery is considered, which underline a high complication rate with high mortality and morbidity rate. This is due to poor nutrition and progression of tumor infiltration (Jaka *et al*., 2013).

Gastric cancer is known as a multifactorial disorder, which involves both environmental and genetic elements. Gastric cancer is the fourth cause of cancer mortality worldwide with a survival rate of about ≤12 months at advanced stage. Young

population has rarely shown the symptoms of this disease and less than 10% of patients that struggle with gastric cancer face the disease before 45 years of age (Pucułek *et al*., 2018).

2.4.4 Treatment of gastric ulcer

Nowadays, several categories of drugs are being used against gastric ulcer disease such as muscarinic M 1 receptor inhibitors; histamine H 2 receptor antagonists and proton pump inhibitors. However, there are side effects associated with these drugs; for example arrhythmia or hematopoietic changes (Almasaudi *et al*., 2016). In addition, the recurrence and refractoriness to treatment are being concerned and leads to the search for a new therapeutic agents (Almasaudi *et al*., 2016; Escobedo-Hinojosa *et al.*, 2018).

2.4.5 Ethanol induced gastric ulcer model

Induction of gastric ulcer by ethanol is a commonly used model in the assessment of potential anti-ulcer drugs. Consumption of ethanol results in the necrosis of tissue, release of inflammatory cells, inhibition of bicarbonate secretion and gastric mucus, reduction of nitric oxide level and blood flow. It also diminish antioxidant enzymatic level, which leads to oxidative damage by increasing the level of MDA (Sistani Karampour *et al*., 2019).

Indeed, the link between alcohol consumption and gastric ulcer development was detected previously. The mechanism of damaging effect of alcohol is not fully understood, however, pro-inflammatory mediators, oxidative stress and apoptosis were identified as main factors involved in the pathogenesis of gastric ulcer upon ethanol consumption. Neutrophils highlight gastric tissue damage by a cytotoxic response that involve the release of MPO. Neutrophils are the key agents in up regulating inflammatory response followed by over secretion of nuclear factor kappa B (NF-κB),

which regulates the release of pro-inflammatory mediators including tumor necrosis factor α (TNF-α). These activities result in amplification of inflammatory cascade by involving secretion of pro-inflammatory mediators and reinforcement of more neutrophil and macrophages activity, thereby intensify gastric ulcer condition (Arab, Salama *et al*., 2015).

2.5 Proteins involvement in tissue injury

2.5.1 Hsp70 protein

Proper cellular function requires proteostasis, which refers to the equilibrium of protein synthesis, folding, trafficking, assembly and degradation. In order to maintain normal cellular function, cells follow strategies to regain this balanced state under stressed conditions. Impaired proteostasis appear as a result of aging or some disease in human. To overcome this complication chaperon molecules, in particular heat shock protein 70 (Hsp70), play a significant role in protein foldings. Hsp 70 in eukaryotic cells support folding and maturation of regulatory proteins. It was reported that Hsp70 do not function as foldases itself, instead it prepare proteins for spontaneous folding and induce unfolding of misfolded proteins followed by proper refolding (Fernández-Fernández & Valpuesta, 2018).

2.5.2 Bax protein

Apoptosis is a natural mechanism occurs during tissue development and aging to maintain homeostasis and cell's population in tissue. This mechanism also activates as a protective process following immune reaction or when cells encounter damages due to diseases or harmful agents (Elmore, 2007). Apoptosis, in addition to its normal developmental function, plays a role in the pathogenesis of a variety of illnesses such as cancer (Hayakawa *et al*., 1999). In addition, apoptosis deregulation leads to diseases

such as peptic ulcer or stomach tumorigenesis (Targa *et al*., 2007), and delay in wound healing (Rai *et al*., 2005). Tissue injury is a defined cause of cellular damage, which may result in the activation of apoptosis process. Certainly, tissue injury leads to molecular response at the cellular level followed by cell death (Aufiero *et al*., 2007).

Bax, is a pro-apoptotic protein that is involved in the process of apoptosis. Under normal physiological condition Bax remain in its inactive form. This protein being activated upon receiving apoptosis signals or external stimulations such as changes in temperature, exposure to hydrogen peroxide or pH alterations. It has been suggested that Bax in its active form induce formation of pores on the outer membrane of mitochondria and facilitate release of cytochrome *c* into cytosol, which triggers initiation of apoptosis pathway. Cytochrome *c* in the inner mitochondrial membrane serves as a key component of electron transport chain, once it's over released into the cytosol it turns on caspases 9, 3 and 7 and results in cell death (Zhang *et al*., 2017). ROS that are generated under several conditions such as inflammation, are known as signaling molecules to mediate apoptosis mechanism and result in cell death (Alarifi *et al*., 2017).

CHAPTER 3: MATERIAL AND METHODOLOGY

3.1 Material

2-pentadecanone was purchased from Sigma-Aldrich Company (USA). For wound healing study this compound was homogenized in 2% CMC. Intrasite gel, which was purchased from University of Malaya's pharmacy, is the standard wound dressing drug (Smith & Nephew Ltd., UK) and it contains 2.3% CMC, 20% propylene glycol as its bioactive compound and water.

For gastro-protective study 2-pentadecanone was mixed with 5% tween 20. Omeprazole, the standard antiulcer drug was also purchased from University of Malaya's pharmacy and used as positive control in gastro-protective study of 2-pentadecanone (Dhiyaaldeen *et al*., 2014; Moghadamtousi *et al*., 2015).

3.2 Animal

Sprague dawley (*SD*) rats were purchased from Animal Experimental Unit (AEU), Faculty of Medicine, University of Malaya. Female *SD* rats (180-250 g) were used in acute toxicity study and male *SD* rats (180-250 g) were used for skin wound healing and gastro-protective experiments. Animals were housed in room temperature (25 ^{0}C) with 12-hour light/dark cycle. Access to food was terminated 24 hours prior starting the experiment, and access to water was eliminated only 2 hours before initiating the experiment. Wound healing and gastro-protective studies were conducted under the approval of Animal Ethic Committee, Faculty of Medicine, University of Malaya (wound healing experiment ethic No: 2019-200108/IBS/R/NAM (2018315) and gastro-protective experiment ethic No: 2016-190819/BMS/R/MAA)). The ethic application for acute toxicity assay was approved by the Animal Ethic Committee under the same ethic numbers mentioned above.

3.3 Toxicity evaluation of 2-pentadecanone

To determine safety of 2-pentadecanone, the instructions given by the Organization for Economic Co-operation and Development (OECD) guideline 423 were followed. Hence, 12 rats were divided into two groups and labeled as control and treatment groups (n=6). Control group was orally given 5% tween 20 (5 mL/kg) and a single dose of 300 mg/kg of 2-pentadecanone was given to the treatment group orally. All animals were fasted a night before starting the experiment with the access to water and received normal diet 4 hours after dosing. An observation period of 14 days was considered to record any abnormal behavioral changes such as ataxia, hypoactivity or hyperactivity as signs of toxicity (OECD 423, 2001). At day 14 rats were euthanized by an overdose of ketamine (300 mg/kg) and xylazine (30 mg/kg) (Kamran *et al*., 2019) and blood was collected by cardiac puncture into EDTA lavender-top tube and sent to a medical laboratory for biochemical analysis. Liver and kidney were also harvested by opening the abdominal cavity with the aid of surgical scissors and specimen holder, fingers where used to detach the organs. Harvested organs were immediately preserved in 10% formalin for histology analysis (Zahra *et al*., 2011).

3.3.1 Biochemistry test of liver and kidney parameters

As drug metabolism occur in liver and its excretion is through kidney (Lakshmanan, 2019), in order to detect the effect of 2-pentadecanone on liver and kidney the following biochemical parameters (Table 3.1) were measured in University Malaya Medical Centre (Diagnostic Laboratory Medicine, UMMC):

Table 3.1: Biochemical parameters were measured to detect the effect of 2-pentadecanone on liver and kidney.

Organs	Parameter
Liver	ALP: alkaline phosphatase, ALT: alanine transaminase, T.protein: total protein, AST: aspartate aminotransferase.
Kidney	sodium, potassium, chloride, CO_2: carbon dioxide, urea, creatinine

3.3.2 Preparation of histology slides

Harvested liver and kidneys after overnight preservation in 10% buffered formalin were processed overnight in automated tissue processing equipment for fixation and dehydration steps (Aplab scientific, SOM-TPROCES-HIS001). Then tissues were embedded in paraffin to prepare blocks for the sectioning process. 5µm sections of the tissue samples were prepared by microtome equipment (Aplab scientific, SOM-TPROCES-HIS001). Prepared sections were stained following Hematoxylin and Eosin (H & E) staining technique adjusted in our lab. H&E staining was initiated by dewaxing the slides in xylene and rehydrating them in alcohol (100%, 95%, 70%) before rinsing with water. Then sections were stained with Hematoxylin stain for 10 minutes and followed by rinsing them with running tap water. Unbound hematoxylin was removed from the sections by rinsing the slides under running tap water prior complete removal of the excess stain with acid alcohol (0.5%), sodium acetate (2%), and alcohol (80%). Then slides were immersed in Eosin stain for 5 minutes followed by alcohol rinse to dehydrate the tissue section and prepare the slides for mounting and cover slipping. The resulting purple color represents nucleus and pink color represent cytoplasm within the tissue (Fischer et al., 2008).

3.4 Wound healing experiment

3.4.1 Excision wound induction

Prior wound creation all rats were anesthetized by using ketamine and xylazine (ketamine: 50 mg/kg and xylazine: 5 mg/kg). Skin of the dorsal neck was shaved by an

electrical shaver and sterilized by 70% alcohol. Wound with 2cm in diameter was created on the shaved dorsal neck area of all groups by the aid of a 2cm round seal and surgical scissors. Any damage to tissue beneath the removed skin was avoided with constant tension of the skin. Created wounds were maintained undressed throughout the experiment (Struck *et al*., 2011; Dhiyaaldeen *et al*., 2014).

3.4.2 Topical wound treatment

For evaluation of wound healing, the rats been divided into four groups: Negative, positive, low dose and high dose. Rats in negative control group treated with 2% CMC, rats in positive control group treated with intrasite gel, meanwhile rats in low dose and high dose groups treated with 10 and 20 mg/ml 2-pentadecanone respectively. Each group consisted of 6 rats and topical application was done twice daily (Dhiyaaldeen *et al*., 2014; Bagheri *et al*., 2018; Cheng *et al*., 2013).

For topical treatment, about 0.2ml of treatment was applied on wound area. Topical treatment was done immediately after wound creation, which considered as day 0 treatment and continued every 5 days for 10 days (El-ferjani *et al.,* 2016).

3.4.3 Measurement of wound closure

Wound closure area (mm^2) was measured at day 5 and 10 using marker and transparent paper. To estimate wound healing percentage the initial and final wound area were applied into the following formula: (Agra *et al*., 2013)

$$\% \text{ wound closure} = A0 - At \,/\, A0 \times 100 \qquad (3.1)$$

Where (A0) is the initial wound area (mm^2) and (At) is the wound area measured at day 5 and 10 (mm^2).

3.4.4 Sample collection

Following the completion of 10 days of the experiment rats were sacrificed by an overdose of ketamine and xylazine. Healed skin and its surrounding was collected and divided into two portions: one portion was kept for tissue homogenate preparation, another portion was kept for histological and IHC analysis (El-ferjani *et al.,* 2016).

3.4.5 Antioxidant assay in skin tissue homogenate: SOD, CAT and MDA measurement

In order to detect antioxidant activity of 2-pentadecanone in skin tissue homogenate, the level of SOD and CAT enzyme's expression was measured. This assay was performed by following the instructions given by Superoxide SOD and CAT assay kits (Cayman Chemical, USA). For both assays healed skin was washed with ice-cold phosphate buffered saline (PBS, PH 7.4) (Saremi *et al.,* 2019).

In preparation for SOD detection, 1 g of tissue was homogenized in 5-10 ml of cold 20 mM HEPES buffer (PH 7.2) by teflon homogenizer (Polytorn, Heidolph RZR 1, Germany). Tissue homogenate was centrifuged at 1,500 g for 5 minutes at 4◦ C. Following the kit instructions 200 µl of the diluted radical detector (ready under this name inside the kit) was added to 10 µl of the supernatant obtained from the tissue homogenate after centrifugation in a 96 well plate.

The reaction was initiated by adding 20 µl of diluted xanthine oxidase into the wells with shaking for 30 minutes at room temperature. Eventually, the absorbance was recorded at 460 nm using a microplate reader (Glomax 9301-010).

To detect CAT expression level within the tissue, 1 g of tissue was homogenized in 5-10 ml of cold buffer (50 mM potassium phosphate, PH 7.0, containing 1 mm EDTA) by using a teflon homogenizer (Polytorn, Heidolph RZR 1, Germany). Tissue homogenate was centrifuged at 10,000 g for 15 minutes at 4∘ C. 20 µl of the supernatant was added to mixture of diluted assay buffer (100 µl) and methanol (30 µl) in the well plate. Reaction was initiated by the addition of 20 µl of diluted hydrogen peroxide to the wells. After 20 minutes incubation at room temperature, the reaction was stopped by adding 30 µl of potassium hydroxide followed by the addition of 30 µl of catalase purpald (chromogen). The plate was incubated again for 10 minutes at room temperature. Next, 10 µl of potassium periodate was added to each well and the plate was incubated for 5 minutes at room temperature with shaking. Eventually, the absorbance was recorded at 540 nm.

To detect MDA level in skin tissue homogenate instructions given by TBARS assay kit were followed (Cayman Chemical) (Moghadamtousi *et al*., 2015). In this assay, 250 µl of RIPA buffer was added into 25 mg of the skin tissue obtained from each group and homogenized using a teflon homogenizer (Polytorn, Heidolph RZR 1, Germany). Next, the tissues were centrifuged at 1,600 g for 10 minutes at 4∘ C. 100 µl of the resulted supernatant of each group was added into separate 5 ml vial, followed by the addition of 100 µl TBA SDS solution. The mixtures were mixed well before the addition of 4 ml of color reagent. These mixtures were boiled for one hour and reaction was stopped by exposure to ice for 10 minutes. Then vials were centrifuged at 1,600 g at 4∘ C followed by incubation at room temperature for 30 minutes. Finally, 150 µl of the prepared tissue samples were loaded into a 96 well plate and the absorbance was recorded at 540 nm.

3.4.6 Histology evaluation of skin tissue: H&E and MT

On the final day of experiment, animals were euthanized by an overdose of ketamine and xylazine. Healed tissue and its surrounding were collected and preserved in 10% buffered formalin to prepare histology slides. For the slide preparation refer to section 3.3.2. Prepared slides were stained following Hematoxylin and Eosin (H & E) as mentioned in section 3.3.2 and Masson Trichrome (MT) staining protocol for gross morphology analysis (Moghadamtousi *et al*., 2015). Purple and pink colors as a result of H&E staining represent nucleus and cytoplasm respectively and green color as a result of MT staining indicates collagen fibers within the skin tissue.

MT staining was performed according to the instructions provided by the kit (Labchem industries Sdn.Bhd. Bulletin No. 100218). The kit contains three ready stains for use (trichrome stain solution A, B, C). Briefly, slides were dewaxed in xylene followed by rehydration through alcohol (100%, 95%, 70%) and rinsed. The slides were stained with hematoxylin for 10 minutes and the excess dye was removed under running tap water. Following this, slides were stained with trichrome solution A, B, and C for 10 minutes. Finally, slides were rinsed with water and dehydrated through alcohol (95%, 100%).

3.4.7 Immunohistochemistry of skin tissue: HSP70 and Bax expression

IHC assay was recommended to perform by other researchers *(Saremi, Bagheri, et al.,* 2019). This assay was performed, by following the instructions given by Polyvalent HRP/DAB detection kit (Abcam, ab64264). In this task peroxidase block had efficiently inhibited endogenous peroxidase, and using protein block eliminated non-specific background. Prepared slides (refer to section 3.3.2) were incubated 3 hours with HSP70 (1:100, Abcam, ab2787) and Bax (1:100, Abcam, ab7977) primary antibodies after dew

axing the slides with xylene, followed by the incubation with anti-mouse and anti-rabbit secondary antibody for 10 minutes. Next, all the slides were incubated with streptavidin peroxidase for 10 minutes followed by the incubation with diaminobenzidine tetrahydrochloride (DAB) chromogen. Finally, enough amount of hematoxylin was added for 1-minute prior mounting. Sections on the slides observed under light microscope, appearance of brown color indicated positive result.

3.5 Gastro-protective study

3.5.1 Induction of gastric ulcer

Firstly, 24 rats were divided into 4 groups (n=6) and were fasted overnight. On the next day all rats received pre-treatment prior ulcer induction. Negative control group was administered with 5% tween 20 (5 ml/kg). Positive control group received 20 mg/kg omeprazole in 5% tween 20 (5 ml/kg). Low dose and high dose groups were pre-treated with 5 ml/kg of 10 mg/kg and 20 mg/kg 2-pentadecanone respectively. All groups received 1 ml of absolute ethanol after one hour of pre-treatment to induce gastric ulcer. One hour after ulcer induction animals were sacrificed by an overdose of ketamine and xylazine. Stomachs were immediately harvested followed by the collection of gastric mucosa into falcon tubes by opening them, then the stomachs preserved in 10% buffered formalin for further analysis (Moghadamtousi *et al*., 2014; Hajrezaie *et al*., 2015).

3.5.2 Gross morphology examination of stomach tissues

Harvested stomachs were examined for gross morphology evaluation. Length and width of the hemorrhagic bands were calculated under a dissecting microscope (1.8x magnification). Ulcer area was estimated by counting the total number of small squares that were covered with the ulcer bands. To calculate ulcer area, the sum of small squares

was multiplied by 4 and 1.8. Inhibition percentage (I %) was also calculated by applying the formula: I % = UA control – UA treated / UA control x 100 (Hajrezaie *et al*., 2015).

3.5.3 Mucus content and acidity measurement

Mucus production in each group was measured by gentle collection of gastric mucosa, followed by weighing it by an electronic balance (Nazarbahjat *et al*., 2016). Gastric juice acidity was measured by collecting the stomach contents into a falcon tube, followed by centrifugation at 3,000 rpm for 15 minutes. A pH meter was used to record the acidity level of the stomach content after being centrifuged (Moghadamtousi *et al*., 2014).

3.5.4 Stomach tissue homogenate preparation

150 mg tissue/ml PBS was homogenized by using a teflon homogenizer (Polytorn, Heidolph RZR 1, Germany). The homogenized sample was then centrifuged (4,000 rpm, 10 minutes, 4 ^{o}C), and the supernatant was collected for the enzymatic level assessment (Moghadamtousi *et al*., 2014).

3.5.5 Antioxidant assay in stomach tissue homogenate: SOD, CAT, NO and MDA measurement

For SOD, CAT and MDA measurement refer section 3.4.5. Nitric oxide (NO) expression level was measured in gastric tissue homogenate by using commercial kit (Cayman Chemical, USA). According to the instructions provided by the kit, stomach tissues from all groups were separately homogenized in PBS, pH 7.4. Tissue homogenate was then centrifuged at 10000 g for 20 minutes. The obtained supernatant from the centrifuged homogenate was filtered through amicon filter (30 kDa molecular weight cut-off filter). 200 µl of assay buffer was added as blank. 80 µl of supernatant

was added into a 96 well plate followed by the addition of 10 µl of enzyme cofactor mixture and 10 µl of nitrate reductase reagent. The plate was covered and incubated for two hours at room temperature. Next, 50 µl of griess reagent R1 was added to each well followed by immediate addition of griess reagent R2. The plate was incubated at room temperature for 10 minutes to allow color development. Eventually, the absorbance was recorded at 550 nm by using a plate reader (Kamran *et al.,* 2019).

3.5.6 Histology evaluation of stomach tissue: H&E and PAS

H&E staining was done following the procedure mentioned earlier in section 3.3.2. PAS staining of the stomach tissues was performed following the instructions given by kit (Bio optica O4-130802). Four solutions "A-E" provided by this kit. Briefly, slides were rehydrated through alcohol (100%, 90%) and distilled water. 10 drops of reagent A, B, and C were added on top of the tissue on slides for 10, 20, and 2 minutes respectively followed by rinsing the slides with distilled water after reagent A and B were added. Next, reagent D was added for 2 minutes with no rinsing prior its addition, however, slides were rinsed after the 2 minutes completed and followed by the addition of Reagent E for 3 minutes and rinsing under tap water for 5 minutes. As a final step, slides were dehydrated with alcohol (Kamran *et al.,* 2019).

3.5.7 Hsp70 and Bax proteins expression level in stomach tissue

This assay was conducted as by following the protocol mentions earlier in section 3.4.8.

3.6 Statistical analysis

All data are reported as mean ± S.E.M. experimental group were compared to negative control group by running One-way ANOVA analysis (SPSS, version 20). $P<0.05$ was considered as significant difference (Saremi *et al*., 2019).

3.7 Images analysis

Image J software, Fiji version was used to analyze the obtained photomicrographs of results. This software measured the intensity of green, purple and brown colors as a result of collagen fiber accumulation in skin tissue, glycogen accumulation in stomach tissue and protein expression in both tissues respectively. The measurements were obtained in term of percentage (Kamran *et al.,* 2019).

CHAPTER 4: RESULTS

4.1 Acute toxicity evaluation of 2-pentadecanone in rats

Acute toxicity assay was performed to test safety of 2-pentadecanone in rats prior conducting wound healing and gastro-protective studies. As a result, animals received 2-pentadecanone showed neither mortality nor signs of toxicity such as diarrhea or bleeding. The values stated in control group for liver and kidney parameters were considered as normal range of the biochemical level for liver and kidney. One-way ANOVA analysis ($p<0.05$) confirmed that there was no significant difference in the biochemical parameters of liver and kidney between the two groups (control and treatment) (Table 4.1 and Table 4.2). Indicating 2-pentadecanone had no effect on the normal function of liver and kidney from biochemical analysis view.

Moreover, H&E assessment of liver (Figure 4.1 A and B) and kidney (Figure 4.1 C and D) of the two groups showed similar microscopic results with no sign of toxicity such as inflammation, fat accumulation and formation of occluded veins in liver, necrosis and cellular swelling in kidney tissue, which has underlined the safety of 2-pentadecanone at dosage of 300 mg/kg in rats.

Table 4.1: Effect of 2-pentadecanone on liver biochemical parameters in *SD* rats.

Group	Albumin (g/L)	ALP (U/L)	ALT (U/L)	GGT (U/L)	T.protein (g/L)	AST (U/L)
Control	36.6±0.2	234.8±26.8	73.6±1.9	2±0.4	58.4±1.4	152±8
300 mg/kg	35.6±0.5	218.4±25.7	74±2.4	1±0.5	55±0.6	168.4±10.9

Abbreviation: ALP: alkaline phosphatase, ALT: alanine transaminase,T.protein:total protein, AST: aspartate aminotransferase. *P<0.05.

Table 4.2: Effect of 2-pentadecanone on kidney biochemical parameters in *SD* rats.

Group	Sodium (mM/L)	Potassium (mM/L)	Chloride (mM/L)	CO_2 (mM/L)	Urea (mM/L)	Creatinine (mM/L)
Control	142±0.4	4.58±0.1	101±0.3	35.2±0.8	7.82±0.2	33.2±0.6
300 mg/kg	141.2±0.6	4.32±0.05	100.6±0.8	34.6±0.8	6.96±0.2	31.6±0.4

*P<0.05

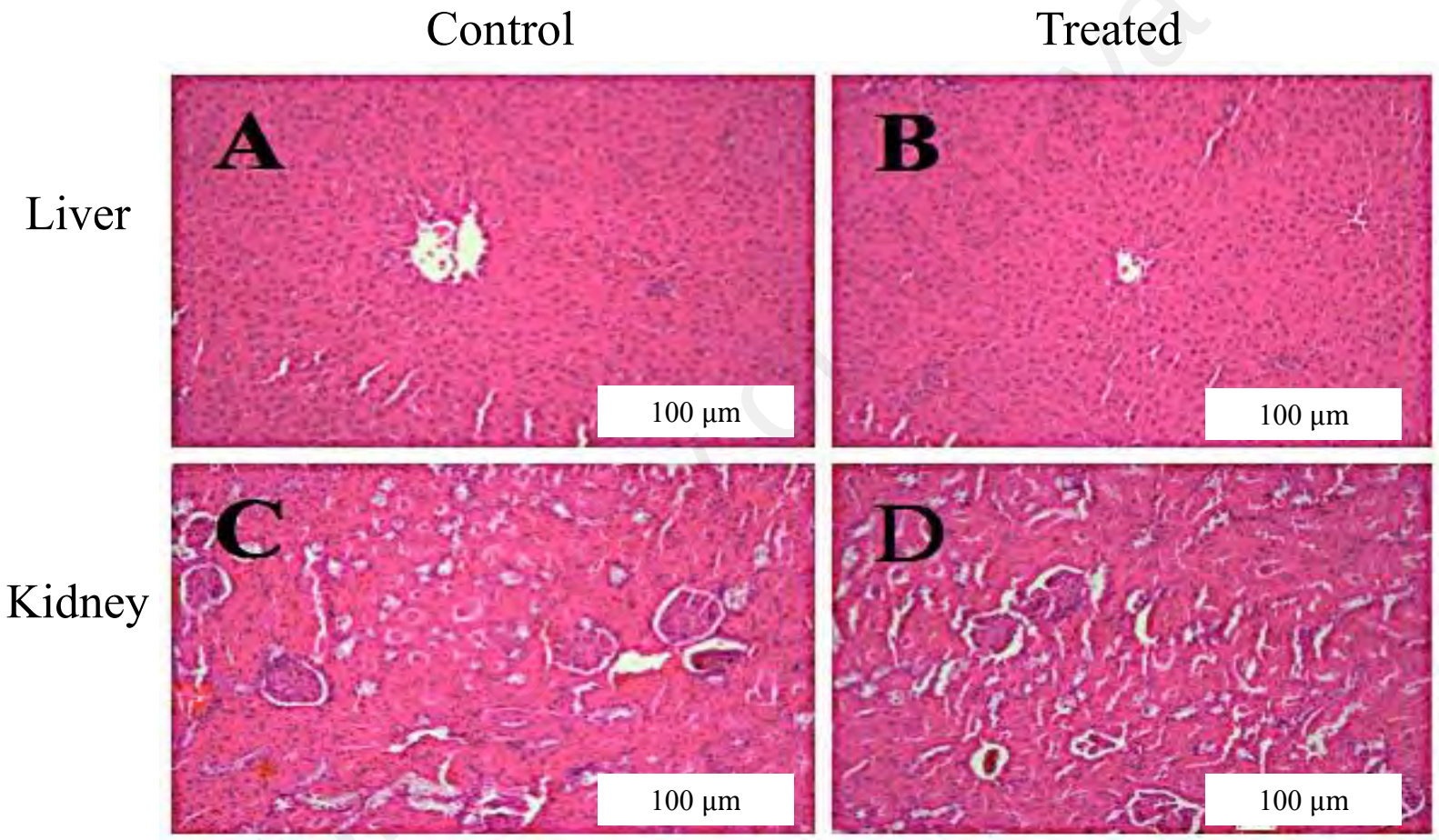

Figure 4.1: Histological sections of liver and kidney. A: liver control (2% CMC), B: liver treatment (300 mg/kg 2-pentadecanone), C: kidney control (2% CMC), D: Kidney treatment (300 mg/kg 2-pentadecanone). Magnification: 20x. Scale bar: 100 µm.

4.2 Evaluation of wound healing effect of 2-pentadecanone

4.2.1 Effect of 2-pentadecanone on skin wound closure

In the evaluation of wound healing effect of 2-pentadecanone, wound areas were recorded at day 0, 5 and 10 and compared to the negative and positive controls (Figure 4.2). This is done to detect how fast 2-pentadecanone can close wounds morphologically and statistically. The results of gross morphology of wound in negative control showed a normal wound with a hard skin surface (dark scab) with no change in the size of the wound after 5 days of treatment (Figure 4.2 a), in comparison to positive control, which showed clear wound healing due to the smaller size of the wound (Figure 4.2 b). Treatment with 2-pentadecanone after 5 days at both doses (Figure 4.2 c, d) showed no obvious wound healing, the appearance of the wounds is more similar to negative control. However, treatment with 20 mg/kg 2-pentadecanone showed reduction in the size of the wound with dehydration. The observations after 10 days of treatment revealed no improvement of wounds in negative control group, with the formation of a rigid dark brown scab on the wounded area (Figure 4.2 a). Similar to day 5, positive control group illustrated almost complete wound healing with hair growth around the wounded area (Figure 4.2 b). Meanwhile, 10 mg/ml 2-pentadecanone after 10 days of treatment showed slow wound healing but better than day 5, and the rigid scab is observed on the wound (Figure 4.2 c). However, 20 mg/ml 2-pentadecanone (Figure 4.2 d) showed comparable wound healing to positive control with closed and more hydrated wound area. These observations are coherent with the results obtained from statistical analysis of wounds in Figure 4.

Statistically, 2-pentadecanone at both doses could significantly (10 mg/ml: $P=0.000$), (20 mg/ml: $P=0.001$) induce wound closure compare to negative control group after 5 and 10 days of treatment (Figure 4.3). Topical treatment of wounds with 2-

pentadecanone (10 mg/ml and 20 mg/ml) and intrasite gel after 10 days resulted in wound closure percentages of 44.16 ± 0.4%, 59.16 ± 0.3% and 65 ± 0.6% respectively (Figure 4.3). These data are far greater than the wound closure percentage of 30 ± 0.8% obtained after 10 days of treatment in negative control group.

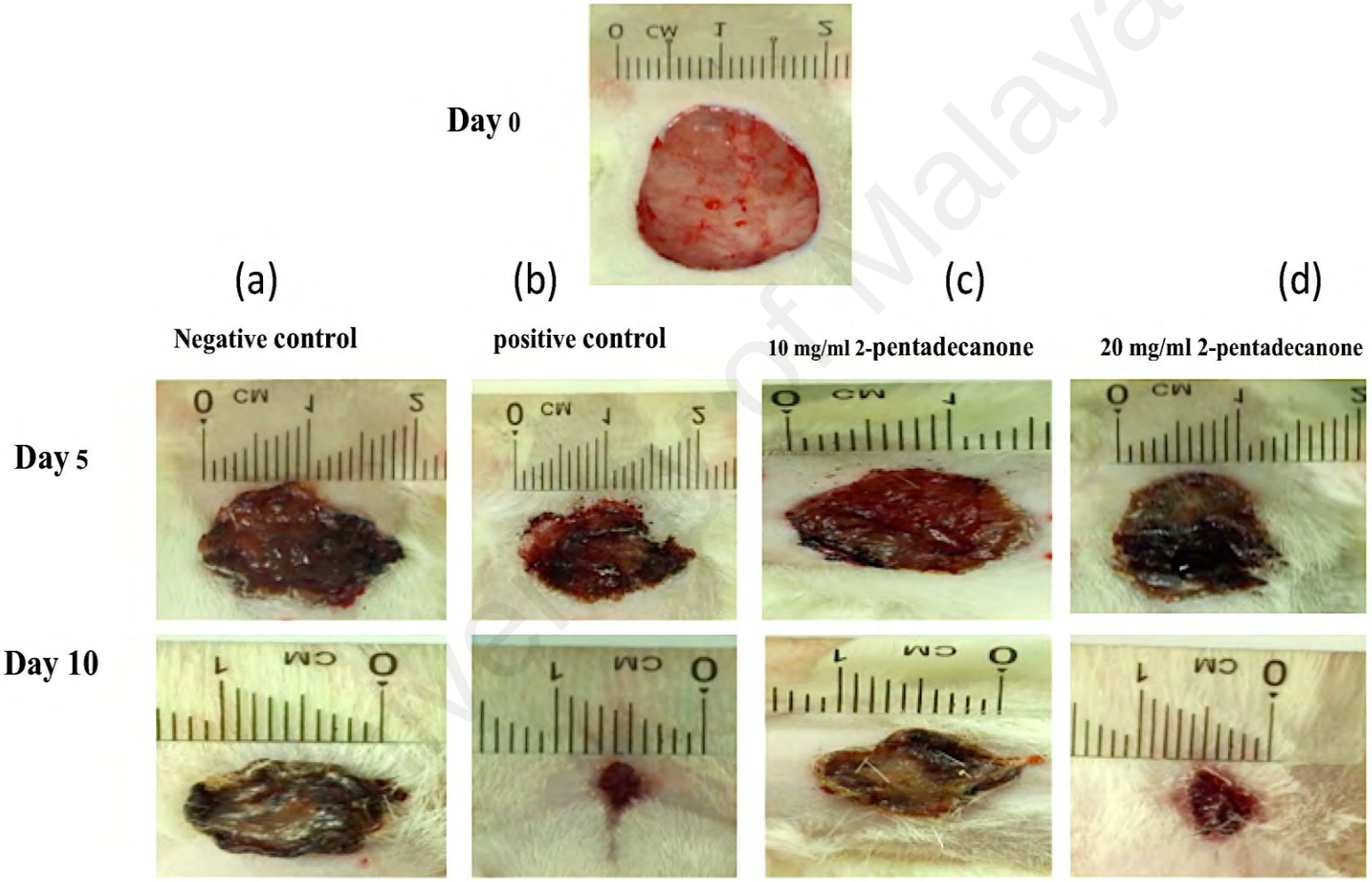

Figure 4.2: Gross morphology evaluation of wounds at day 0, 5 and 10. a: negative control (2% CMC), b: positive control (intrasite gel), c: 10 mg/kg 2-pentadecanone, d: 20 mg/kg 2-pentadecanone.

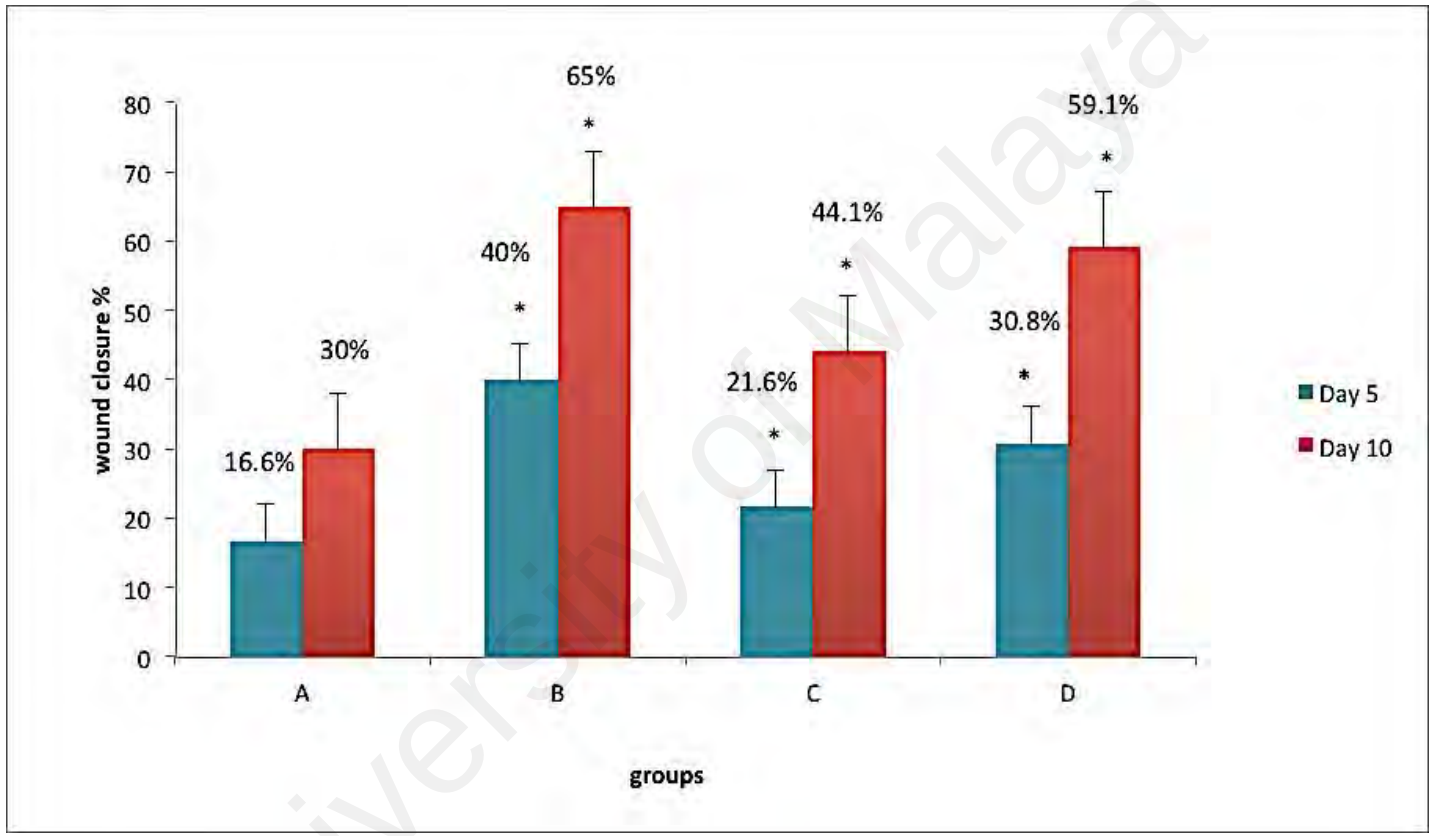

Figure 4.3: Effect of 2-pentadecanone on percentage of wound closure in rats. A: negative control (2% CMC), B: positive control (intrasite gel), C: 10 mg/kg 2-pentadecanone, D: 20 mg/kg 2-pentadecanone. Data are reported as means ± SEM of six animals per group. *P<0.05.

4.2.2 Effect of 2-pentadecanone on antioxidants enzyme expression in skin tissue homogenate

In order to determine antioxidant activity of 2-pentadecanone in skin tissue homogenate, expression level of SOD and CAT enzymes were measured in this experiment. Basically, normal wound healing mechanism is associated with the release of ROS follow by oxidative damage (Bickers & Athar, 2006). Therefore, antioxidant enzymatic activity in wounded tissue is necessary to scavenge free radicals and diminish the damaging effect on ROS on cells (Moghadamtousi *et al*., 2015). The results of these measurements are reported in Table 4.3. Topical treatment with 2-pentadecanone (10 and 20 mg/ml) and intrasite gel resulted in significant elevated activity of SOD (P=0.01) and CAT (P=0.00) enzymes in the wound tissue compared to negative control, which is due to a protective response of 2-pentadecanone and intrasite gel against oxidative damages.

Excessive release of ROS during wound healing mechanism results in lipid peroxidation (Moghadamtousi *et al*., 2015). This condition inhibit the normal wound healing mechanism (Altavilla *et al*., 2001). Therefore, in this experiment, we measured MDA level (Table 4.3) as a biomarker of oxidative damage. The results revealed significant (P=0.00) attenuation of lipid peroxidation product (MDA) within the granulation tissue of treated groups with positive control and 10 and 20 mg/ml 2-pentadecanone in comparison to negative control group. This result strongly suggests that 2-pentadecanone has exerted antioxidant activity at both doses that could positively affect the wound healing process in the rats.

Table 4.3: Effect of topical treatment on antioxidant enzymatic expression level *P<0.05.

Groups	SOD (U/ml)	CAT (nmol/min/ml)	MDA (U/mg)
Negative control	12.41±0.00	52.39±0.02	51.22±0.01
Positive control	63.25±0.05*	135.20±0.00*	12.56±0.01*
10 mg/ml 2-pentadecanone	29.08±0.03*	75.11±0.03*	28.56±0.00*
20 mg/ml 2-pentadecanone	54.91±0.02*	114.54±0.02*	17.89±0.01*

Data are reported as means ± SEM of six animals per group. A value of *P<0.05 was considered significant. Abbreviations: CAT, catalase, SOD, superoxide dismutase, MDA, malonialdehyde.

4.2.3 Histology evaluation of wounded skin tissue after 10 days of treatment

In this assay H & E staining was used to view different layers of epidermal tissue (epidermis and dermis) under microscope (Agarwal & Krishnamurthy, 2019), and MT stain was used to detect collagen formation in skin tissue (Costa *et al*., 2019).

The result of H & E staining in Figure 4.4, (4x magnification) showed great scar width with ruptured epidermis and dermis layer in negative control group (Figure 4.4 A), while wounds treated with positive control (Figure 4.4 B), 10 mg/ml 2-pentadecanone (figure 4.4 C) and 20 mg/ml 2-pentadecanone (Figure 4.4 D) showed reduced scar width with intact epidermis and dermis layers.

Moreover, 40x magnification of the same results (Figure 4.4) showed the granulation tissue in each group, where huge amount of inflammatory cells and RBC observed in negative control group (Figure 4.4 A) in comparison to positive control (Figure 4.4 B) and treated groups with both doses of 2-pentadecanone (Figure 4.4 C and D).

In comparison, the reduced intensity of inflammatory cells in groups treated with 10 mg/ml and 20 mg/ml of 2-pentadecanone (Figure 4.4 C and D) and the similarity of this observation to the positive control group (Figure 4.4 B).

Collagen synthesis within granulation tissue examined by MT staining (Figure 4.5), moreover, red blood cells (RBC) and inflammatory cells were also detected in this assay. Negative control group (Figure 4.5 A) showed less covered area with collagen fibers (green color), high number of RBC (red color) and inflammatory cells (purple) in the skin tissue, which displays that inflammatory phase did not proceeded to the next phase after 10 days of treatment in comparison to treated groups (Figure 4.5 B, C and D). These observations were coherent with the analysis of collagen deposition percentage in each group (Figure 4.6). Intensity of green colored collagen was measured by image J software (Fiji version), which enabled us to detect collagen percentage. The least collagen deposition percentage (9.7%) belongs to negative control group, positive control, 10 mg/ml 2-pentadecanone and 20 mg/ml 2-pentadecanone showed collagen deposition percentages of 57.8%, 30.3% and 44.3% respectively. As a result topical treatment with 2-pentadecanone had a comparative effect to positive control, however high dose of this compound showed closed collagen deposition percentage to positive control.

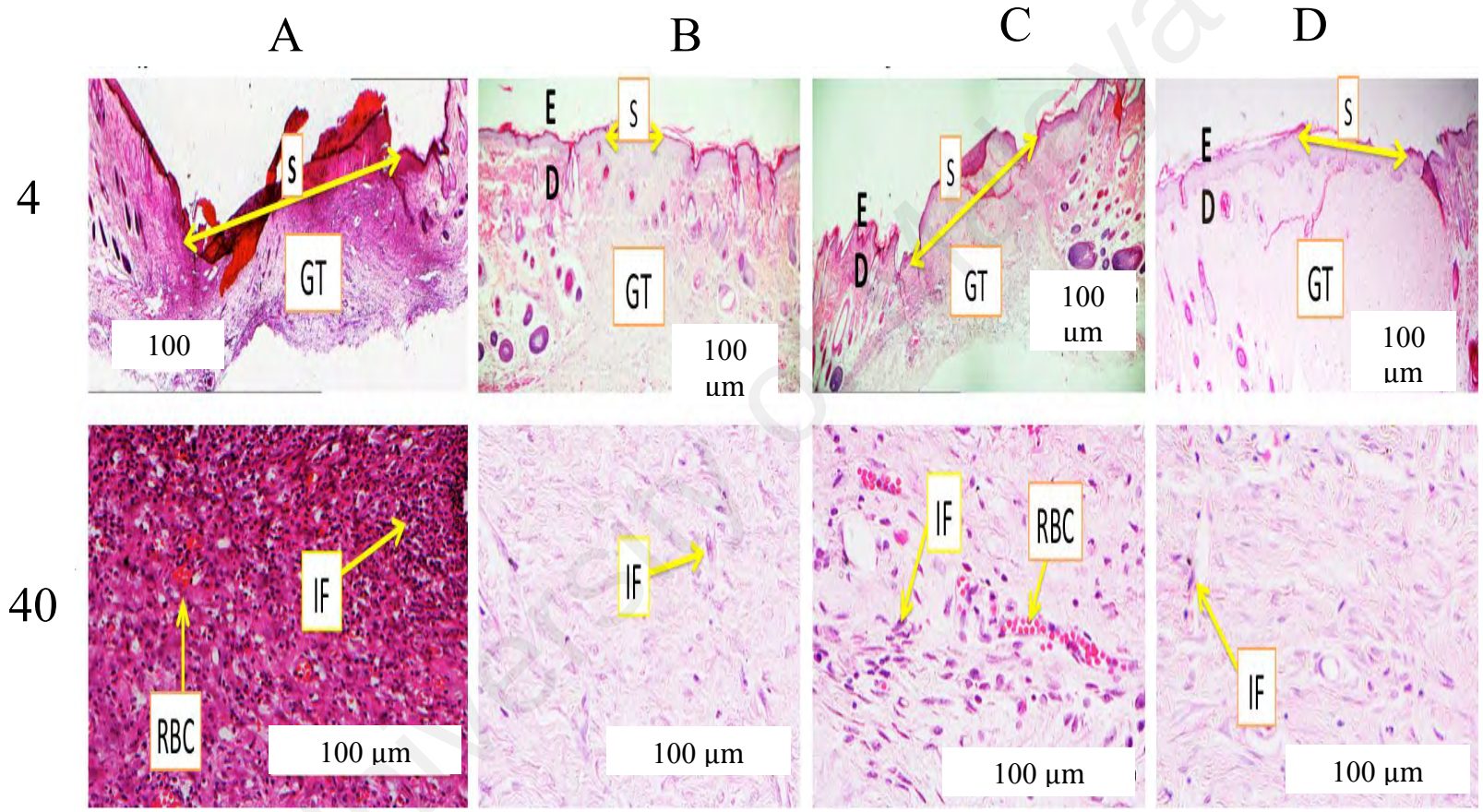

Figure 4.4: Histological analysis (H&E) of wounds on day 10 at two magnifications (4x magnification and 40x magnification). A: negative control (2% CMC), B: positive control (intrasite gel), C: 10 mg/ml 2-pentadecanone, D: 20 mg/ml 2-pentadecanone. Abbreviations: S, scar. E, epidermis. D, dermis. GT, granulation tissue. IN, inflammatory infiltration. RBC, red blood cells. Scale bar: 100 µm.

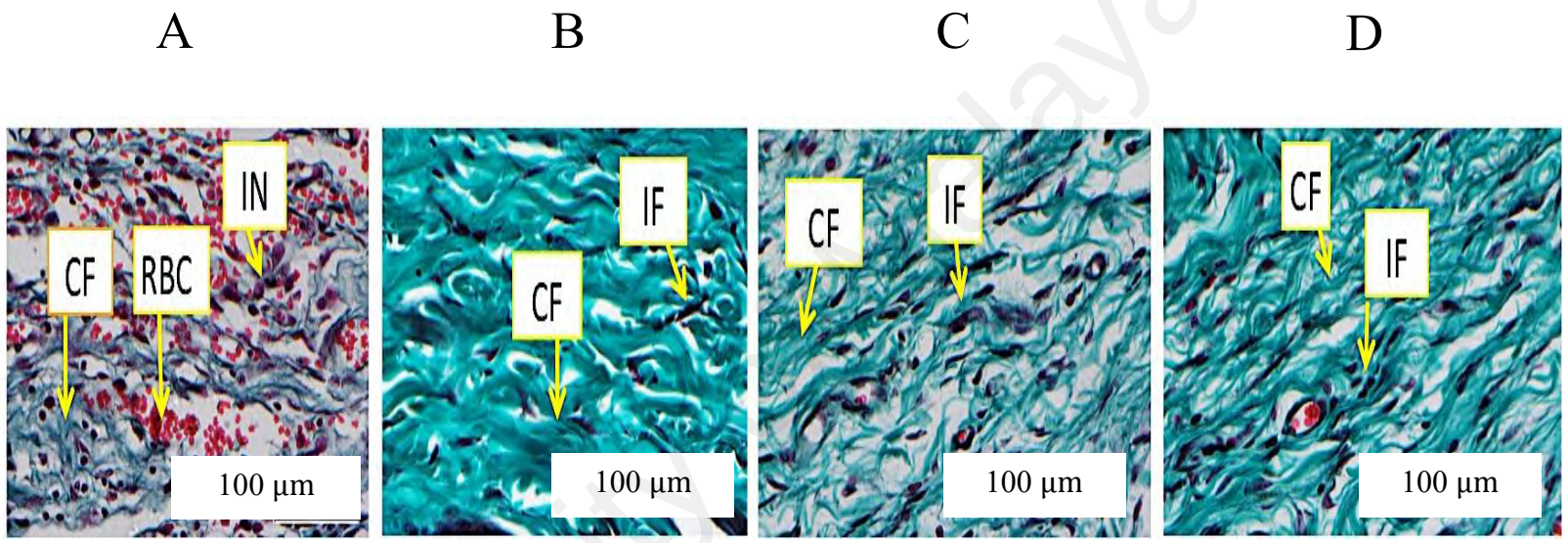

Figure 4.5: MT analysis of wounds on day 10 at 40x magnification. A: negative control (2% CMC), B: positive control (intrasite gel), C: 10 mg/ml 2-pentadecanone, D: 20 mg/ml 2-pentadecanone. Abbreviations: S, scar. CF, collagen fiber. RBC, red blood cell. Scale bar: 100 µm.

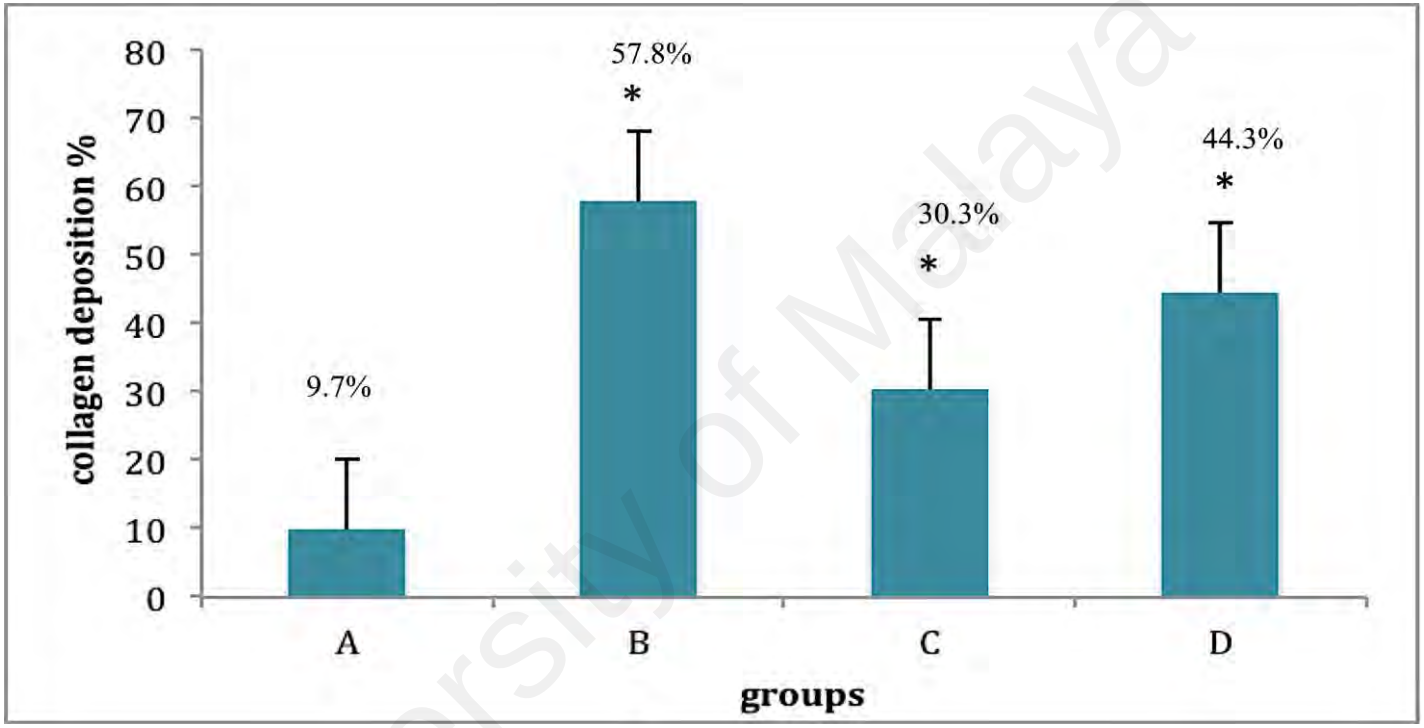

Figure 4.6: Collagen deposition percentage in granulation tissue after 10 days of treatment. A: negative control group (2% CMC). B: positive control group (intrasite gel). C: treated with 10 mg/ml 2-pentadecanone. D: 20 mg/ml 2-pentadecanone. *P<0.05.

4.2.4 Effect of 2-pentadecanone on HSP70 and Bax proteins expression in skin tissue

HSP70 is known to support the wound healing process (Atalay *et al*., 2009), while Bax protein results in generation of ROS and death of cells in a wounded tissue (Rouhollahi *et al*., 2015). Therefore, in this experiment, the expression level of Hsp70 was measured as a supportive element in wound healing process and Bax as a biomarker of cellular damage in wounded skin tissue.

Protein expression analysis was done by performing IHC assay to detect the expression level of HSP70 and Bax proteins in skin tissue sections after 10 days of topical treatment. Brown color is an indicator of HSP70 and Bax expressions within the granulation tissue after 10 days of treatment.

In the analysis of HSP70 expression level (Figure 4.7 A) faint brown color is observed, which is the result of insignificant HSP70 expression in granulation tissue of negative control. While this observation (brown color) is significantly intense in positive control group (Figure 4.7 B) and groups treated with two doses (10 and 20 mg/ml) of 2-pentadecanone (Figure 4.7 C and D). The same observational record was done for Bax protein in granulation tissue, where negative control group (Figure 4.7 A) revealed extreme brown color as a result of excessive Bax protein expression. Reduced brown color intensity was observed in positive control group (Figure 4.7 B) and treated groups with both doses of 2-pentadecanone (Figure 4.7 C and D), however high dose of 2-pentadecanone showed more comparable color intensity to positive control group. These observations were statistically analyzed and the results (Figure 4.8) showed compatibility to our observations. As a result, 18.9% HSP70 protein expression level was detected in negative control group. This percentage was higher in positive control

group (59.4%) and high dose and low dose of 2-pentadecanone groups (28.2% and 41.6% respectively). Moreover, 55.1% Bax protein expression level was detected in negative control group, while this percentage reduced to 15.0% in positive control group and 30.2% and 22.8% in low dose and high dose 2-pentadecanone groups respectively.

Statistical analysis supported that 2-pentadecanone has significantly stimulated over expression of HSP70 (P=0.00) and significantly inhibited Bax expression (P=0.00) within the tissue. Intensity of brown color was measured by image J software (Fiji version).

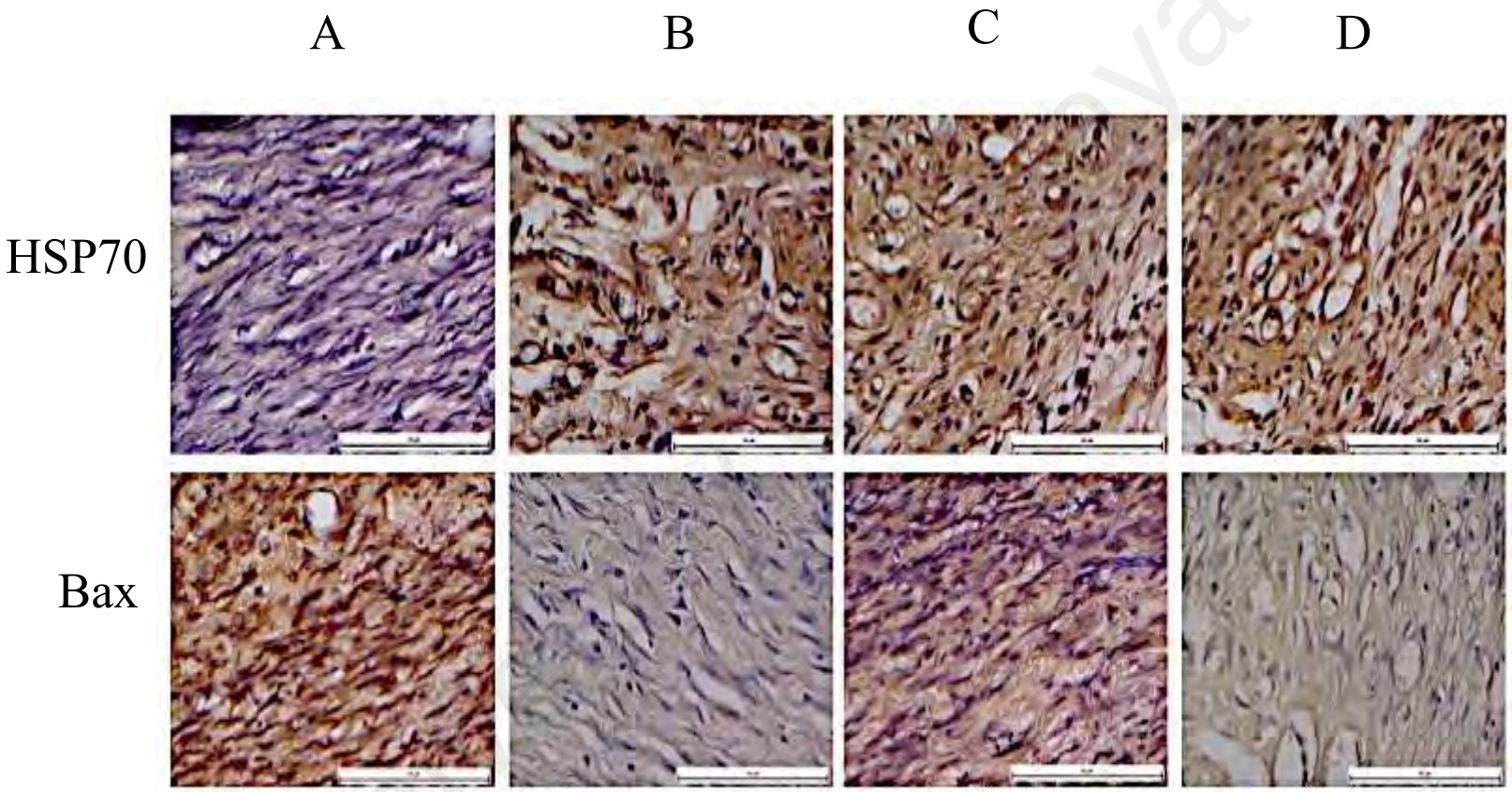

Figure 4.7: Immunohistochemical analysis of Hsp70 and Bax expression in granulation tissue from 4 groups of rats. A: negative control (2% CMC). B: positive control (intrasite gel). C: 10 mg/ml 2-pentadecanone. D: 20 mg/ml 2-pentadecanone. Scale bar: 100 µm.

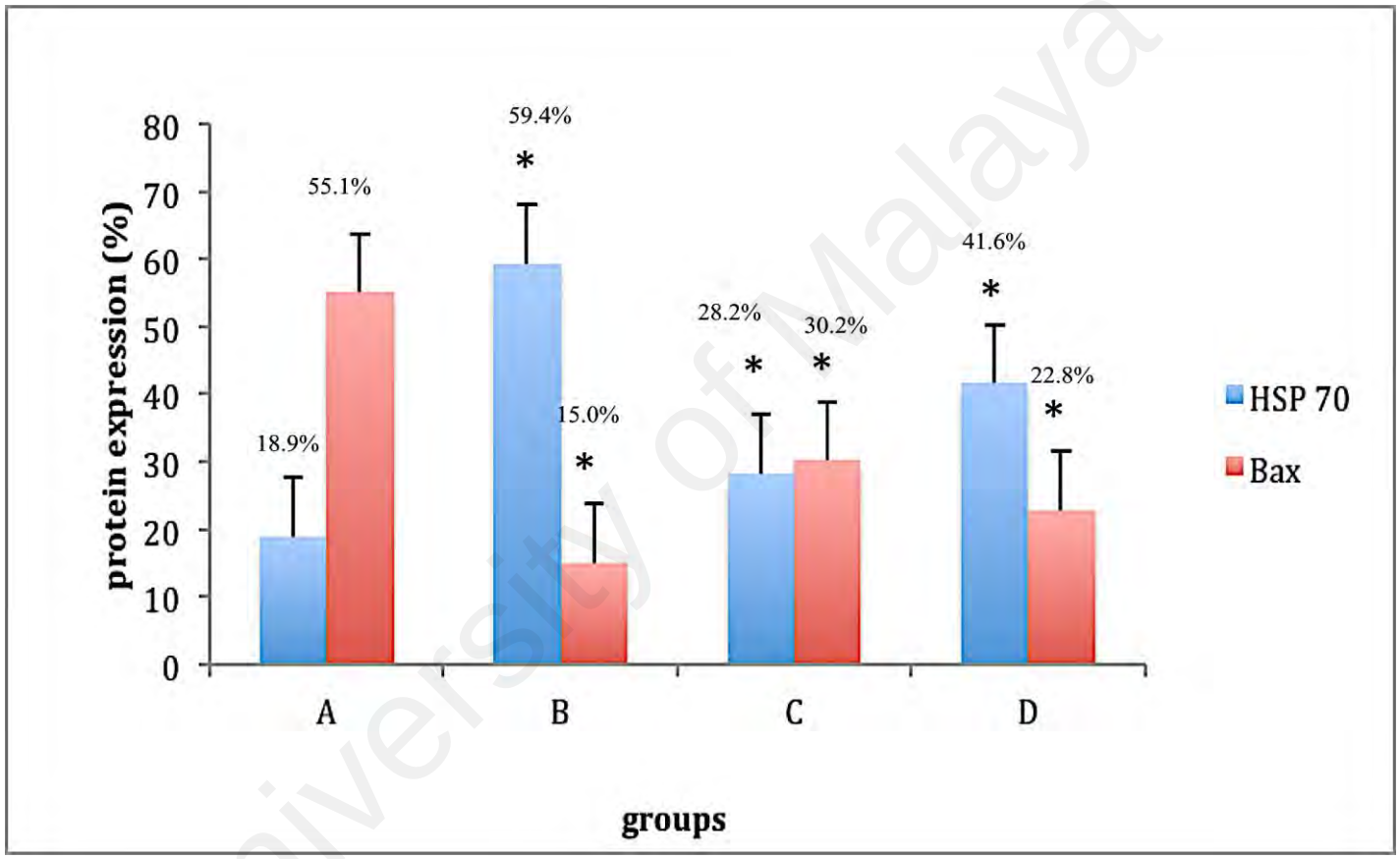

Figure 4.8: Percentage of Hsp70 and Bax expression in granulation tissue (Image J analysis of IHC) after 10 days of treatment. A: Negative control (2% CMC). B: positive control (intrasite gel). C: 10 mg/ml 2-pentadecanone and D: 20 mg/ml 2-pentadecanone. *P<0.05.

4.3 Evaluation of gastro-protective effect of 2-pentadecanone

4.3.1 Gross morphology of stomach tissues

In the gross morphology evaluation of gastro-protective effect of 2-pentadecanone against ethanol induced ulceration, stomach tissues were collected, dissected and observed to detect the development of hemorrhagic bands as a sign of stomach ulcer (Moghadamtousi *et al*., 2014). Gross morphology of negative control group (Figure 4.9 A) showed a great formation of hemorrhagic bands (lesions formed in red color), while Pre-treated groups with omeprazole and 2-pentadecanone (Figure 4.9 B, C and D) showed significant inhibitory effect on the formation of hemorrhagic bands in comparison to negative control group.

Moreover, flattening of the folds is observed in groups received omeprazole and 2-pentadecanone at both doses (Figure 4.9 B, C and D). Ulcer area and inhibitory effect (calculated by the formula mentioned in methodology section 3.5.2) of positive control and 2-pentadecanone are shown in Figure 4.10 Omeprazole, low dose and high dose of 2-pentadecanone inhibited ulcer formation by 90.18%, 80.58% and 86.63% accordingly. Statistical analysis has revealed significant (P=0.00) ulcer inhibitory effect in groups received 2-pentadecanone compare to negative control.

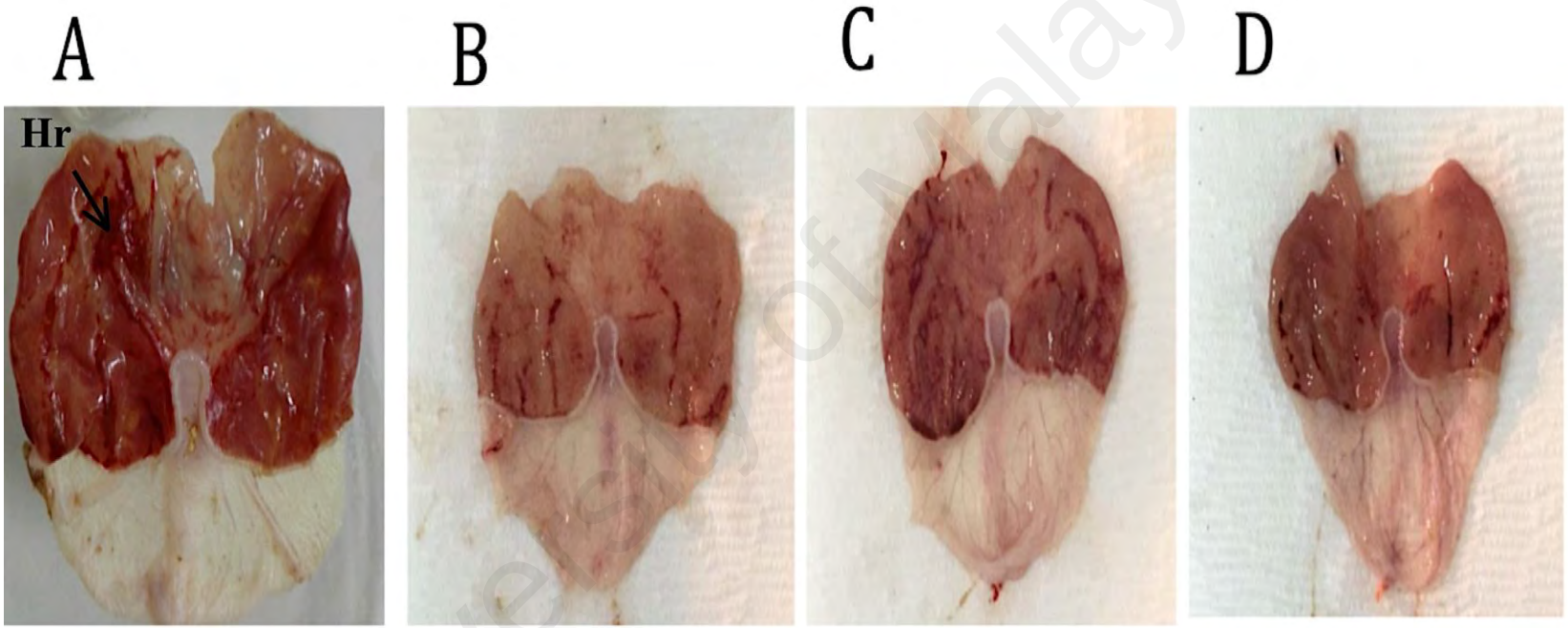

Figure 4.9: Gross morphology of stomach tissues. A: negative control (5% tween 20). B: positive control (20 mg/kg omeprazole). C: 10 mg/kg 2-pentadecanone. D: 20 mg/kg 2-pentadecanone. Hr: hemorrhagic bands.

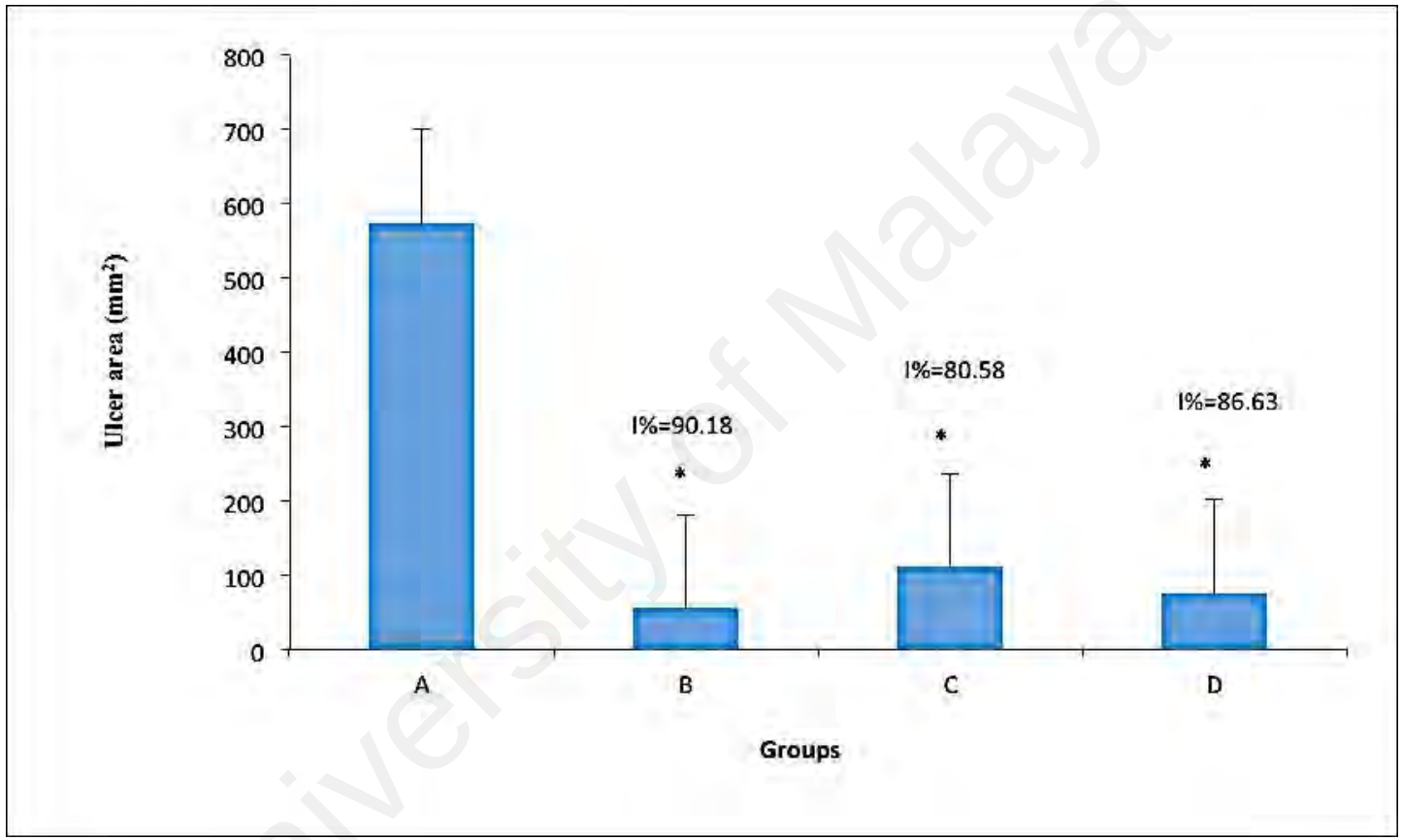

Figure 4.10: Effect of 2-pentadecanone on ulcer area and inhibition percentage. A: negative control (5% tween 20). B: positive control (20 mg/kg omeprazole). C: 10 mg/kg 2-pentadecanone. D: 20 mg/kg 2-pentadecanone. I%: inhibitory percentage. *P<0.05.

4.3.2 Evaluation of 2-pentadecanone effect on acidity and mucus secretion levels in stomach

In order to detect protective effect of 2-pentadecanone, acidity and mucus secretion level in the stomach tissue were measured. As a result of this assay, negative control group which received no pre-treatment showed a very low pH level (strong acidic environment) and mucus weight (low protective agent). These measurements increased in positive control group and groups pre-treated with both doses of 2-pentadecanone (10 and 20 mg/kg). All the measurements are represented in Table 4.4. The result of statistical analysis supported the gastro-protective effect of 2-pentadecanone against damaging effect of ethanol (P=0.00) by lowering the acidity level of stomach juice and inducing higher mucus production.

Table 4.4: Effect of 2-pentadecanone on pH level and mucus secretion.

Groups	pH	Mucus weight (g)
A	2.3±0.2	0.5±0.1
B	6.8±0.3*	2.7±0.1*
C	5.7±0.4*	2.0±0.1*
D	6.5±0.3*	2.3±0.2*

Abbreviations: A: negative control (5% tween 20). B: positive control (20 mg/kg omeprazole). C: low dose 2-pentadecanone (10 mg/kg) and D: high dose 2-pentadecanone (20 mg/kg). *P<0.05.

4.3.4 Effect of 2-pentadecanone on antioxidant enzyme expression, MDA production and NO accumulation in stomach tissue homogenate

The antioxidant activity of 2-pntadecanone in stomach tissue homogenate is reported in table 4.5. Ethanol has greatly reduced the antioxidant activity in negative control group. Groups that received omeprazole and 2-pentadecanone at both doses revealed significant elevation of SOD (P=0.008), notable raise in CAT activity (P=0.00), significant increase in NO synthesis (P=0.004) and remarkable decrease in MDA production level (P=0.00).

Table 4.5: Effect of 2-pentadecanone on antioxidant enzymatic expression and NO level.

Groups	SOD (U/mg)	CAT (nM/min/ml)	MDA (µM/g)	NO (µM/g)
A	6.9±0.00	62.5±0.01	31.2±0.00	2.9±0.00
B	29.7±0.00*	140.0±0.02*	9.8±0.00*	11.2±0.00*
C	21.8±0.00*	120.0±0.03*	15.2±0.00*	8.9±0.00*
D	24.4±0.00*	130.9±0.00*	12.5±0.00*	10.1±0.00*

Data are reported as means ± SEM per group. Abbreviations: SOD: superoxide dismutase, CAT: catalase, NO: nitric oxide, MDA: malonialdehyde. A: negative control (5% tween 20). B: positive control (20 mg/kg omeprazole). C: low dose 2-pentadecanone (10 mg/kg) and D: high dose 2-pentadecanone (20 mg/kg). A value of *P<0.05 was considered significant.

4.3.5 Histology examination of stomach tissues

Microscopic characterizations of stomach tissues are illustrated in Figure 4.11 and 4.12. Sever damage of gastric mucosa with submucosal edema are observed in negative control group (Figure 4.11 A). Pretreatment with positive control and 2-pentadecanone (low and high dose) resulted in reduction in inflammation and RBC (Figure 4.11 B, C and D). These observations are in consonance with the gross morphology analysis.

Glycogen deposition in gastric epithelium was detected by PAS staining. Magenta color represents positive result. Negative control group (Figure 4.12 A) showed extremely faint magenta color in comparison to other groups, this emphasizes on negligible glycogen production in stomach tissue. Groups that received 2-pentadecanone at 10 and 20 mg/kg (Figure 4.12 C and D) showed similar glycogen production level to positive control group (Figure 4.12 B). These observations were analyzed by Image J software (Fiji version) (Figure 4.13). Statistical analysis has confirmed the significant positive effect of 2-pentadecanone on glycogen production (P=0.00).

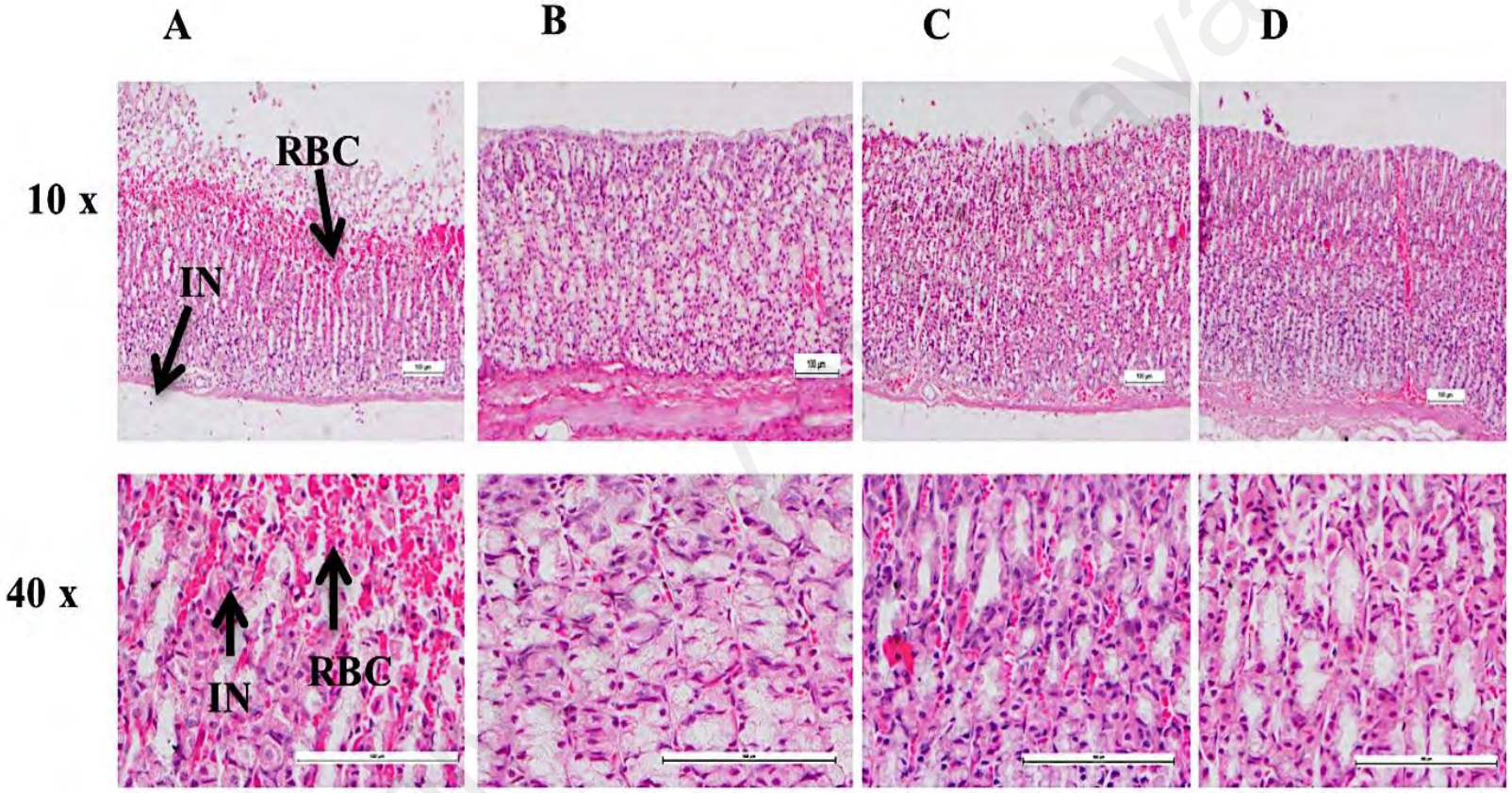

Figure 4.11: Evaluation of the gastric tissue stained with H & E stain. A: negative control (5% tween 20). B: positive control (20 mg/kg omeprazole). C: low dose 2-pentadecanone (10 mg/kg) and D: high dose 2-pentadecanone (20 mg/kg). IN: inflammatory cells, RBC: red blood cells. Scale bar: 100 µm.

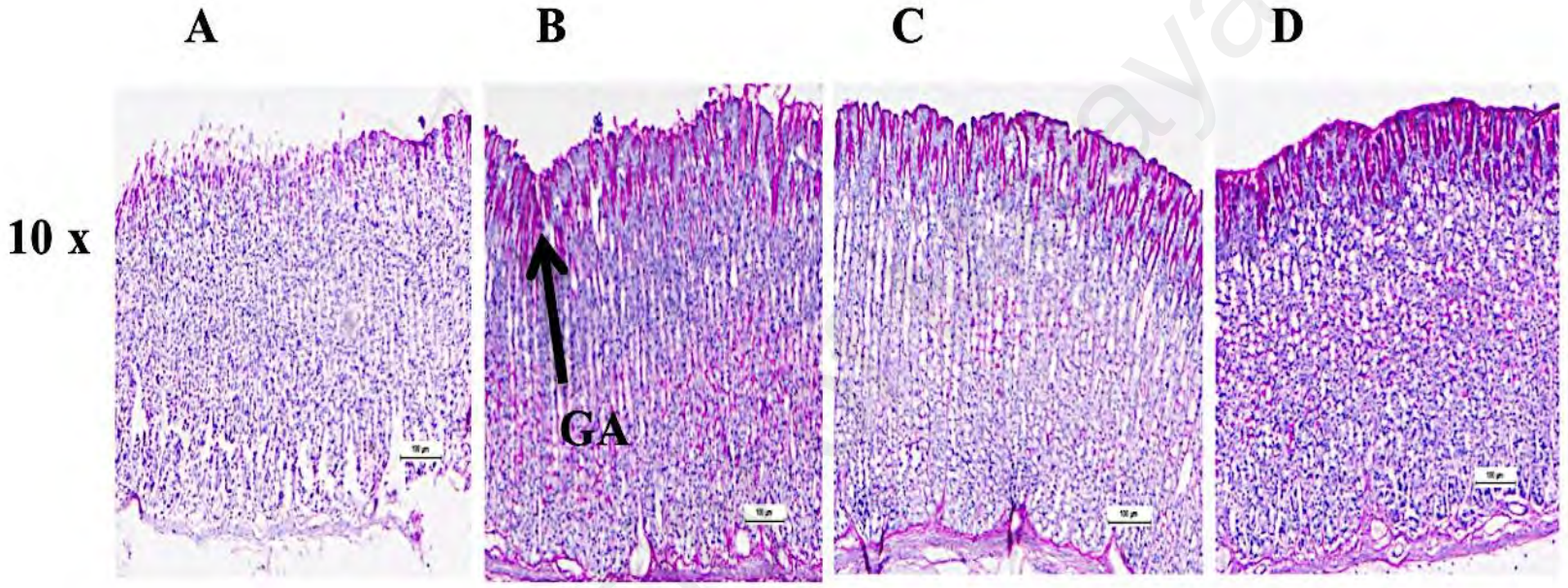

Figure 4.12: Effect of 2-pentadecanone on glycogen accumulation in stomach tissue as a result of PAS staining. A: negative control (5% tween 20). B: positive control (20 mg/kg omeprazole). C: low dose 2-pentadecanone (10 mg/kg) and D: high dose 2-pentadecanone (20 mg/kg). GA: glycogen accumulation. Scale bar: 100 µm.

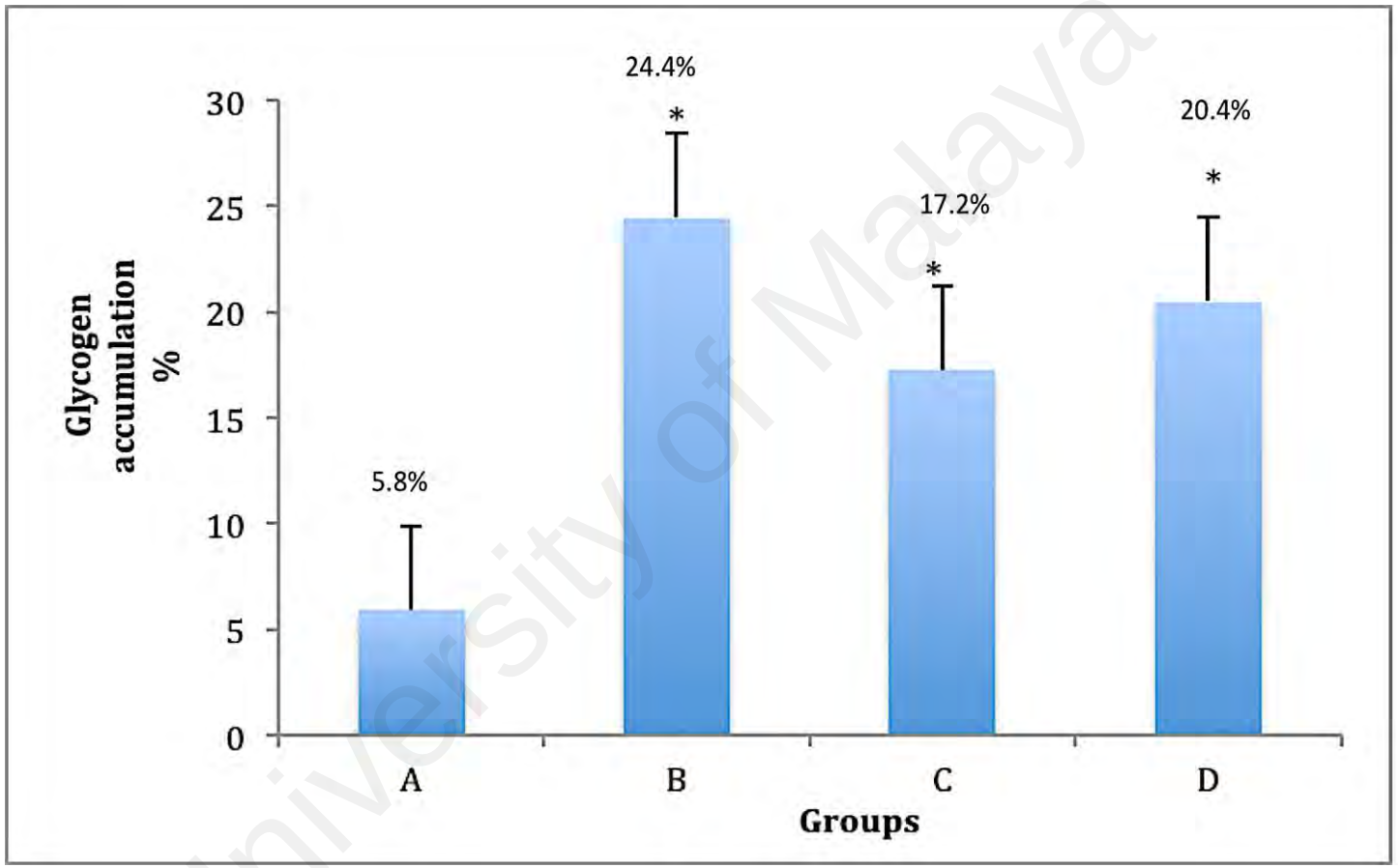

Figure 4.13: Glycogen accumulation percentage in stomach tissue. A: negative control (5% tween 20). B: positive control (20 mg/kg omeprazole). C: low dose 2-pentadecanone and D: high dose 2-pentadecanone.

4.3.6 HSP70 and Bax proteins expression level in stomach tissue

To confirm the defensive effect of 2-pentadecanone against gastric ulcer formation the expression of two main proteins were assessed through IHC assy. The outcomes of this assay revealed up regulation of Hsp70 protein and down regulation of Bax protein in gastric mucosa of pre-treated groups with low dose and high dose of 2-pentadecanone (Figure 4.14 C and D), and these results are comparable to the positive control group (Figure 4.14 B). Negative control group showed the least Hsp70 accumulation and highest Bax expression in gastric mucosa (Figure 4.14 A). These results were analyzed in a more comparative manner by Image J software (Fiji version), and the outcome of this analysis is shown in Figure 4.15. Statistical analysis of these results showed significant positive effect of 2-pentadecanone on Hsp70 and Bax proteins expression (P=0.00).

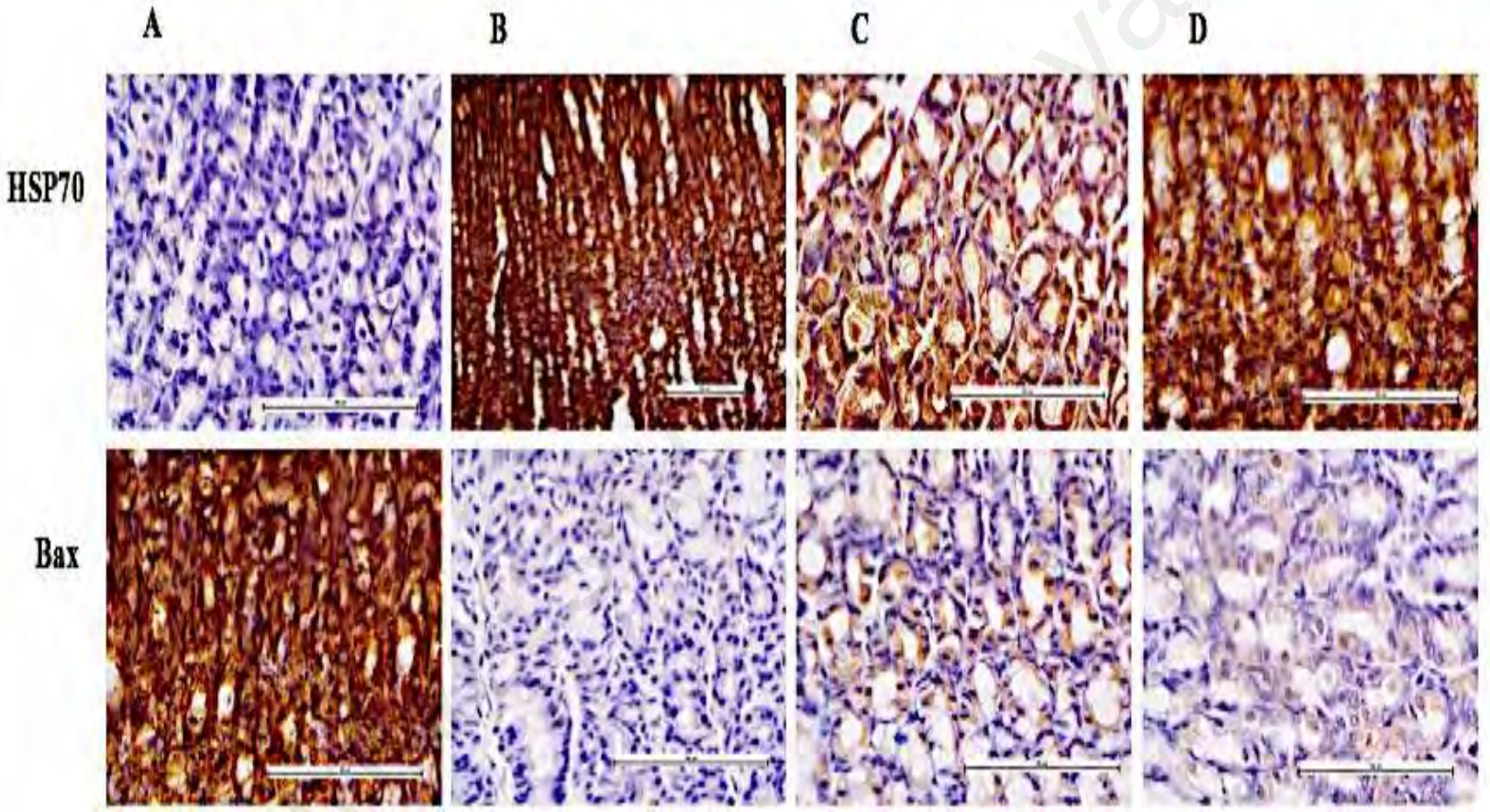

Figure 4.14: Immunohistochemical analysis of HSP70 and Bax expression in stomach tissue. A: negative control (5% tween 20). B: positive control (20 mg/kg omeprazole). C: low dose 2-pentadecanone (10 mg/kg) and D: high dose 2-pentadecanone (20 mg/kg). Scale bar: 100 µm.

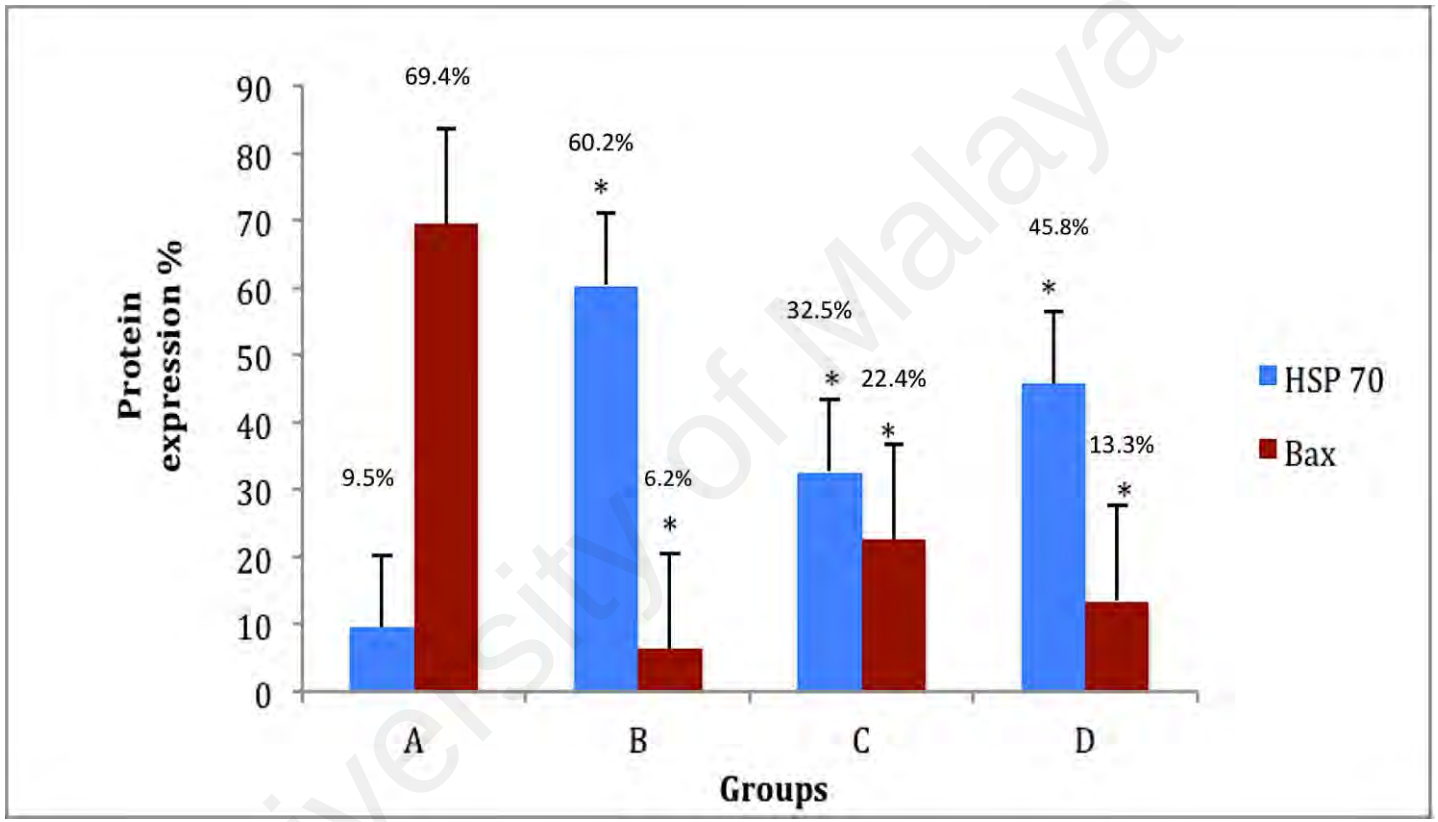

Figure 4.15: Effect of 2-pentadecanone on Hsp70 and Bax expression in stomach tissue. A: negative control (5% tween 20). B: positive control (20 mg/kg omeprazole). C: 10 mg/kg 2-pentadecanone. D: 20 mg/kg 2-pentadecanone. *P<0.05.

CHAPTER 5: DISCUSSION

In this study 2-pentadecanone was tested as a new potential drug for its skin wound healing and gastro-protective effects after its safety was confirmed through acute toxicity assay. Promising results were obtained which are discussed in this section.

5.1 Safety verification of 2-pentadecanone through acute toxicity analysis

Toxicity evaluation of any potential drug is necessary prior testing its biological activity. Signs such as mortality, behavioral changes of animal (ataxia, hypoactivity or hyperactivity), microscopic morphology alterations and abnormality in the level of liver and kidney functional parameters offer damaging effect of the tested compound (Moghadamtousi *et al*., 2014). Acute toxicity is a type of toxicity test which employs biochemical and histological analysis to detect safety of a compound (Arome & Chinedu, 2014). Early detection of the potential effect of a substance to develop hepatotoxicity (liver toxicity) and nephrotoxicity (kidney toxicity) is of the utmost importance for health risk evaluation. Liver is highly exposed to drug toxicity as drug metabolism and biotransformation of chemical compounds occur in liver via enzymatic activity of microsomal cytochrome P450 monooxygenases (CYP450). Usually, the products of biotransformation of chemicals are biologically inactive molecules, however productions of toxic reactive metabolites are detected in some cases. Production of these toxic metabolites are the main reason for nephrotoxicity, as kidneys play a significant role in the excretion of metabolites (Pizzo *et al*., 2015).

In this study, we have assessed and examined biochemical parameters of liver and kidney and also the histology of these two organs to confirm safety of 2-pentadecanone. Recommendation provided by the OECD guideline 423 has stated that females are physiologically more sensitive to drug toxicity. With the recommendation, female rats

were used in acute toxicity assay conducted in the current study. In this assay, control group was used as a reference for comparison of the results after treatment with 2-pentadecanone (OECD 423, 2001).

Laboratory liver tests are useful in the detection of liver dysfunction. Some of the liver enzymes and proteins are sensitive to the abnormalities occur by exposure to various agents, therefore they are used as biochemical markers of liver dysfunction (Gowda *et al*., 2009). In this study, we targeted albumin, ALP, ALT, GGT, T. protein and AST as the biochemical markers of Liver dysfunction, where the results (Table 4.1) showed no significant difference in the level of these biomarkers between the control group and treated group with 2-pentadecanone. This indicates 2-pentadecanone caused no toxicity and liver dysfunction. To look at each biochemical parameter of liver individually, albumin is a biomarker of liver cirrhosis, which is the 14th most common reason of death. Progressed liver cirrhosis is detectable by a decrease in the level of plasmatic albumin, which results in impaired liver function. Predominantly, albumin protein is a remarkable prognostic agent, introduced as a significant predictor of mortality in patients with liver cirrhosis (Carvalho & Machado, 2018). The analysis of albumin level in the current study showed no sign of liver cirrhosis in the treated group with 2-pentadecanone as the level of plasmatic albumin did not deviated significantly in comparison to control group. ALP, a biomarker of liver injury, raise upon liver damage (Hasan *et al*., 2018). The elevation of this enzyme is associated with cholestatic disease in liver, which can appear as a reason of DILI (Newsome *et al*., 2018). In this study 2-pentadecanone showed its safety as control group and treated group with 2-pentadecanone revealed similar range of ALP plasmatic level.

ALT and AST enzymes are significant indicators of liver dysfunction. These enzymes are found in liver and activates in response to liver injury. An increase in the level of these enzymes is the most significant sign of liver dysfunction detected during biochemical test profile. ALT is found in tissues other than liver, however it is considered as exclusive indicator of liver illness since non-liver enzymatic elevation is rare (Newsome *et al*., 2018). As a result of the current study, the plasmatic level of ALT and AST showed no significant increase in group treated with 2-pentadecanone in comparison to control group. This confirms the safety profile of 2-pentadecanone. Another important enzyme is GGT, which is an abundant enzyme found in liver and it is useful in the detection of liver injury. Elevation of this enzyme is common in patients with liver dysfunction as a result of alcohol consumption or injury induced by drugs. GGT is one of the most significant predictors of liver death (Newsome *et al*., 2018). In this study, GGT was considered as a biochemical marker for DILI, where the results showed no significant increase or decrease in the level of this enzyme after treatment with 2-pentadecanone in comparison to control group. The similar range of GGT in both groups confirmed safety of 2-pentadecanone.

Bilirubin, the breakdown product of haeme, found either in unconjugated or conjugated forms. Under normal condition, insoluble unconjugated form of this protein is transported to liver, where it is converted into a soluble conjugated form for excretion. Most laboratories report the level of total protein, which refer to unconjugated and conjugated forms. Elevation of either form result in a rise in the measurement of total protein (T.protein). Remarkably, liver disease result in hyperbilirubin, which in turn lead to high level of T.protein (Newsome *et al*., 2018). The results of T.protein analysis in this experiment, revealed similar range of the protein in control group and treated group with 2-pentadecanone. This is due to the non-toxic

effect of 2-pentadecanone, which did not affect the normal concentration of T.protein in blood plasma.

In addition, in this experiment the level of sodium, potassium, chloride, CO_2, urea and creatinine were evaluated as biochemical markers of kidney dysfunction. Kidneys are organs that take the responsibility of waste and toxins excretion such as urea and creatinine and also regulation of electrolytes volume (Stewart & Pasha, 2018). Drugs are common agents lead to kidney injury, where the interaction of toxic products with nephrons may cause nephrotoxicity (kidney toxicity) (Perazella, 2018). Electrolytes, urea and creatinine are some biochemical markers considered as indicators for kidney dysfunction. Electrolyte test include sodium, potassium and chloride. An elevation in the level of electrolytes, specifically, potassium is the most remarkable complication of kidney dysfunction (Gowda *et al*., 2010). The results of this experiment (Table 4.2) showed very close measurements in the level of electrolytes (sodium, potassium and chloride) between the control group and treated group with 2-pentadecanone. Safety of 2-pentadecanone can be confirmed by having no deviation in the level of electrolytes after treatment with 2-pentadecanone.

Urea, the other biochemical marker of kidney failure, is the end product of protein and amino acids catabolism. It is produced by liver and travel through intracellular and extracellular fluids. Basically, kidneys filter urea out of blood. Concentration of urea in serum is the most significant indicator of kidney failure, where elevated level of urea in blood serum is a sign of kidney dysfunction. It indicates blockage of urinary tract, dehydration and bleeding in the digestive tract (Gowda *et al*., 2010). As a result of urea analysis in the current study, urea level was not elevated in the response to 2-pentadecanone in comparison to control group; in fact, its level was detected close to

the urea level in control group.

Creatinine, another biochemical marker of kidney dysfunction, is the end product of creatinine phosphate breakdown in muscles. This product is commonly used as a biochemical marker for the detection of kidney dysfunction. Kidney failure is expected when creatinine level is increased in blood serum. Creatinine measurement may not be constant as its level is affected by muscle function, muscle composition, activity and diet (Gowda *et al*., 2010). In the present study, the level of creatinine in treated group with 2-pentadecanone was compared to the control group, which considered representing the normal range of biomarker, as a result there was no significant difference between the creatinine level of the two groups. Again, this is an indication of the safety of 2-pentadecanone. Moreover, kidneys play a major role in preservation of acid-base homeostasis. In patients with kidney disease, kidney lose its ability to excrete daily acid load, resulting in metabolic acidosis (accumulation of acids such as ammonium) (Raphael, 2019). Metabolic acidosis is a common issue associated with advanced kidney disease. This condition results in a reduction in renal bicarbonate production therefore, it result in less concentration of bicarbonate and carbon dioxide (CO_2) (Adamczak *et al*., 2018). The obtained results of this study, showed no significant decrease in the level of CO_2 after treatment with 2-pentadecanone in comparison to control group. This result represents safety of 2-pentadecanone in rats.

In addition to support these findings, the histology of liver and kidney (Fig 4.1) after 14 days of treatment showed no significant difference in comparison to control group. As it was mentioned earlier in result section, the histology of liver illustrated no abnormalities such as hepatic necrosis, acute and chronic hepatitis, acute and chronic cholestasis, granulomatous hepatitis, steatosis and hepato-portal sclerosis. Also, the

histology of kidney illustrated no signs of toxicity such as interstitial edema, tubular epithelial cells death, formation of vesicles in epithelial cells and ablation of tubular epithelium from the basement membrane. As results of biochemical and histology analysis of liver and kidney in this study, safety profile of 2-pentadecanone was confirmed following results of acute toxicity assay in female rats leading to confidently conducting wound healing and gastric ulcer prevention experiments.

5.2 The enhancing effect of 2-pentadecanone on skin wound healing

In this study, topical treatment was performed as antibacterial activity of 2-pentadecanone was suggested previously (Gunalan *et al*., 2016). In the present study, considering the potential wound healing effect of 2-pentadecanone, full thickness excisional wound model was applied. Untreated wounds in negative control group were detected by the development of a dark, rigid scab (Figure 4.2 A). This is an indication of dehydrated skin surface, which slow down the process of wound healing (Thomas Hess, 2011). The observation of the treated wounds suggested that topical application of 2-pentadecanone facilitated wound healing mechanism in animals by eliminating the formation of the dark scab, and reducing the size of wounds after 10 days of treatment (Figure 4.2 D).

Wound area measurement offers details about the progress of healing, helps to determine and decline the likelihood of unsuccessful therapies (Gethin, 2006). Myofibroblasts are the cells that induce wound contraction (Chitturi *et al*., 2015). Upon an injury, inflammation signals activate fibroblast cells and encourage development of proto-myofibroblasts. TGF-β1 stimulates proto-myofibroblasts to differentiate into myofibroblasts and express α-SMA, which is a contractile protein. α-SMA induce contractile forces followed by induction of intracellular tension via utilizing ATP to

slide actine filaments toward each other and result in wound closure (Li & Wang, 2011). In this research wound area was measured after 5 and 10 days of treatment (Figure 4.3) to confirm the effect of topical administration of 2-pentadecanone on closure of wounds. Our findings, suggested that 2-pentadecanone has a promising effect on induction of wound closure, as figure 4.3, has clearly shown the wound closure after 10 days of treatment with 2-pentadecanone.

Inflammation is a critical phase in wound healing mechanism. Basically, this phase induces immune response and attack invading pathogens and remove dead tissue. Although inflammation is crucial, it must be resolved after injury to achieve successful wound repair (Yeh *et al*., 2017).

Past studies have reported that the number of infiltrated macrophages into the site of injury rise at day 3 of wounding and persists until day 7, neutrophils number at the wound site grows 12 hours after wounding, and decrease on day 3 (Maeda *et al*., 2011; Mori *et al*., 2008; Wang *et al*., 2016; Makino *et al*., 2014). In the present study the number of inflammatory cells was not estimated on different days, however different study showed a decline in the number of inflammatory cells after 2 days and 7 days of diabetic wound treatment with 2-pentadecanone in comparison to negative control group (Siyumbwa *et al*., 2019). The results of histology examination in the present study, illustrated that inflammatory cell infiltration was intense in the negative control group and the intensity (purple color) dropped after 10 days of topical treatment with positive control and 2-pentadecanone at both doses (Figure 4.4). This is an indication of prolonged inflammatory phase in negative control group, which display slow wound healing progress within 10 days of topical treatment. In contrast, 2-pentadecanone

accelerated wound healing mechanism within 10 days of treatment, which is comparable to the outcome of treatment with positive control.

The potential wound healing effect of 2-pentadecanone (10 and 20 mg/ml) was also evidenced by the formation of a new thin epidermis layer in treated groups (Figure 4.4), which is considered as a defensive layer to avoid further injury via covering the whole wounded site (Moghadamtousi *et al*., 2015). In addition, a significant reduction in the scar length of treated groups was observed (Figure 4.4), which support the positive effect of the tested compound. However, topical treatment of wound with high dose (20 mg/ml) of 2-pentadecanone showed to be more effective in both gross morphology evaluation (Figure 4.2) and H&E histology section (Figure 4.4 D). The lack of new epidermal layer development and the high number of inflammatory infiltrations in negative control group (Figure 4.4 A) represents prolonged presence of inflammation. Similarity of the observations in the treated groups to the positive control group (Figure 4.4 B, C and D) can be a proof for the wound healing effect of 2-pentadecanone.

In addition to H&E analysis of skin tissue, MT analysis was also done to detect collagen deposition in skin tissue (Suvik, 2012). Collagen is the main constituent of extracellular dermal matrix, and its role is associated with the scar formation during the wound healing process (Rangaraj *et al*., 2011). Collagen deposition is necessary for the skin integrity and tensile strength of skin (Nyström, 2016). In the present study, the microscopic findings (Figure 4.5) revealed well-organized deposition of collagen fibers and less inflammatory cells in treated groups with 2-pentadecanone (10 and 20 mg/ml) compared to negative control group. The histology and microscopic assessment indicated that topical treatment of the wounded tissue with 2-pentadecanone has accelerated skin tissue recovery and regeneration.

Hsp proteins are associated with wound healing process, and released under various cellular stress conditions. Hsp70 is reported as a major stress-inducible Hsp protein and it plays anti-apoptotic and pro-proliferative activities (Zhang *et al*., 2019). This protein was reported to support wound healing process via playing role in cellular proliferation, collagen formation, regulation of inflammation and wound debridements removal (Atalay *et al*., 2009). Hsp proteins are maintained at their minimum level under normal physiological condition. Stressed conditions such as injury, stimulates expression of this protein to a higher level to attenuate inflammation followed by acceleration of wound healing mechanism (Bruemmer-Smith *et al*., 2001). Hence, any disturbance in the expression and operation of Hsps under stressed condition results in impaired wound healing (Moghadamtousi *et al*., 2015). Hsp70, among the subclasses of heat shock proteins, was reported to be responsible for the cell survival and protein homeostasis within the wound bed (Wagstaff *et al*., 2007). Our results, obtained from IHC analysis, demonstrated that up-regulation of Hsp70 upon treatment with 2-pentadecanone is comparable with the influence of positive control. This finding remarkably showed the inducible effect of 2-pentadecanone on the expression of Hsp70 protein within the wound bed, which supported the improvement of wounded tissue. This finding is also in consonance with other wound healing studies, which have suggested up-regulation of Hsp70 as a result of treatment could improve the healing process (Bagheri *et al*., 2018), (Rouhollahi *et al*., 2015), (Saremi *et al*., 2019).

A relationship between Bax expression protein and apoptosis in wound healing process was suggested previously. Apoptosis is a critical mechanism that affects progression of wound healing process negatively. Inflammatory cells are detected as the initial cells that migrate to the site of injury to inhibit microorganism's activity and mediate inflammatory response. These active cells result in the generation of ROS in

the wounded tissue, and activate apoptosis process in the surrounding cells and keratinocytes. This activity is due to the release of pro-apoptotic proteins such as Bax, which affect mitochondria and empower further progression of cellular damage (Bagheri *et al*., 2018; Rouhollahi *et al*., 2015; Saremi, Bagheri *et al*., 2019).

In this study, Bax expression analysis in granulation tissue was declined in the skin tissues treated with 2-pentadecanone (10 and 20 mg/ml) and intrasite gel compared to negative control group (Figure 4.7 and 4.8). This observation and analysis were compatible with other wound healing studies, where suggested that, a potential drug with wound healing effect decreased Bax expression in granulation tissue and improved wound healing process

The wound healing mechanism is associated with skin ischemia, a condition that supports development of ROS as a result of leukocytes activity within the wounded tissue. The higher generation of free radicals triggers more activated leukocytes, which in turn amplifies the damaging effect of free radical in wound site via oxidative damage (Bickers & Athar, 2006). Under normal physiological situation body homeostasis maintain the level of free radicals at a balance state via the influence of endogenous antioxidant enzymes. When the level of free radicals exceed the antioxidant enzymes level, it cause some structural modifications and cellular injuries (Valko *et al*., 2007), (Soneja *et al*., 2005). Antioxidant protective mechanism of cells function via enzymatic and non-enzymatic scavengers. SOD, CAT, glutathione-s-transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPX) are enzymes that considered as enzymatic antioxidants defensive line of cells, that have a crucial effect in inhibition of oxidative stress resulted from the activity of ROS (Moghadamtousi *et al*., 2015).

In this study we have determined SOD and CAT expression level and MDA production level in the skin tissue homogenate to detect antioxidant activity of this compound. Our results have strongly suggested that topical treatment of wounds using 2-pentadecanone (10 and 20 mg/ml) induced a high antioxidant capacity in the wounded tissue, which hasten wound healing mechanism (Table. 4.3). Initially, the protective mechanism against free radicals is mediated by SOD, which diminishing oxidative damage via scavenging superoxide anion (O^{-}_{2}) and results in the generation of reactive hydrogen peroxide (H_2O_2). Next, CAT plays a crucial role to convert H_2O_2 into water and oxygen (Szuster-Ciesielska *et al*., 2004). Cellular deposition of H_2O_2 leads to the formation of hydroxyl radicals (OH^-) through fenton reaction, which empower oxidative damage (S. Park & Imlay, 2003), (Bai & Cederbaum, 2001). In addition, over production of ROS and oxidative damage in wounded tissue activate lipid peroxidation process (Moghadamtousi *et al*., 2015). This is due to the reaction of free radicals with lipids, which generate MDA as a final product. Detection of MDA is a sign of insufficient antioxidant activity (Nagamani *et al*., 2015). Lipid peroxidation process accounts for the impairment of endothelial cells, fibroblast and collagen metabolism, and keratinocyte capillary permeability. Excessive lipid peroxidation in wound tissue result in a defect in expression of VEGF protein and consequently impairment in wound healing process (Altavilla *et al*., 2001).

5.3 Gastro-protective effect of 2-pentadecanone against ethanol induced ulceration

Stomach ulcer disease was reported in as a prominent illnesses around the world (Ahmed, 2019). High number of studies have been reported their efforts on testing potential effect of whole plant extracts (Rouhollahi *et al*., 2014; Park *et al*., 2019; AbdulSalam & Hassan Kha, 2019). In the present research the gastro-protective activity

study involved the effect of 2-pentadecanone as a pure synthetic compound, since pharmaceutical industries usually focus on synthetic compounds as drug discovery sources (Atanasov *et al*., 2015). However, there are studies tested the gastro-protective effect of a single compound rather than plant extracts, and obtained promising results (Nazarbahjat *et al*., 2016; Sistani Karampour *et al*., 2019; Sánchez-Mendoza *et al*., 2019). But, the effective doses of these compounds were reported far higher (100 and 200 mg/kg) than the effective doses of the available drug in the market (20 mg/kg omeprazole). However, in the current study the doses of 2-pentadecanone did not exceed the dose of omeprazole, which are 10 and 20 mg/kg of the compound.

Despite the fact that proton pump inhibitors (PPIs) are commonly prescribed for the treatment of gastric ulcer, there are side effects such as iron and vitamin B12 deficiency and bone fracture associated with the consumption of these drugs (Kinoshita *et al*., 2018). Hence, exploring new, safe and effective therapeutic agent is necessary. This study has offered promising results, suggesting 2-pentadecanone has gastro-protective activity.

Gastric ulcer is evidenced by the formation of hemorrhagic bands in the stomach (Moghadamtousi *et al*., 2014). The gross morphology observation in this study showed the resulting hemorrhagic deterioration was clearly diminished by pre-treatment with 10 and 20 mg/kg of 2-pentadecanone with less hyperemia (Figure 4.9 C and D), which is comparable to the suppressive effect of positive control on the formation of hemorrhagic bands (Figure 4.9 B). In addition, microscopic observation revealed that 2-pentadecanone pre-administration (10 and 20 mg/kg) resulted in a remarkable ulcer inhibitory percentage compared to negative control (Figure 4.10).

Moreover, a great mucosal surface area in pre-treated groups is evidenced by flattening of the mucosal folds (Figure 4.9 B, C, D). Flattenings of the folds were observed in groups received omeprazole and 2-pentadecanone at both doses, this indicates muscle relaxation, which promotes greater mucosal surface area and results in the higher exposure to the tested compound (Halabi *et al*., 2014). To compare the present research to the latest study conducted on the gastro-protective effect of zingerone in 2019 (Sistani Karampour *et al*., 2019), 2-pentadecanone resulted in a greater mucosal area.

Proton pump inhibitors are highly used to reduce gastric acid secretion (Li *et al*., 2004) and protect mucosal layer (Schneeweiss *et al*., 2006). Gastric mucosa defensive effect of proton pump inhibitors such as omeprazole and their acid inhibitory activity are the main reason for their healing properties. Thus any potential agent with the similar activities may plausibly offer promising antiulcer effect (Moghadamtousi *et al*., 2014). Therefore, as a next step of this study we determined gastric juice acidity and mucus secretion in rats.

Over production of gastric acid in patients with gastric ulcer can amplify the gastric injury, which reversibly affect production level of mucus. This imbalanced situation between the aggressive and defensive factors develops a condition of hyperacidity. In such condition, proton pump inhibitors suppress acid secretion to recover production of mucus (Moghadamtousi *et al*., 2014). In this study, administration of ethanol as a necrotizing agent has dropped pH and mucus secretion level, while these measurements increased in pre-treated groups with 2-pentadecanone and omeprazole (Table 4.4.). This result revealed that 2-pentadecanone recovered the loss of gastric mucus in pre-treated rats under both doses. Yet, positive control showed slightly higher defensive effect by

representing the highest pH and mucus secretion level among all the measurements (Table 4.4.).

In order to confirm the potential effect of 2-pentadecanone in relation to our study, its effect on antioxidant enzymatic and NO expression level in stomach tissue homogenate was detected. ROS such as O^{-}_{2} and H_2O_2 release as a result of neutrophil infiltration and play role in gastric mucosa oxidative damage and tissue destruction (Lim *et al*., 2019). To defense body against the harm, antioxidants provide protection against ROS by maintaining them at their physiological level, thus prevent tissue damage(Avci *et al*., 2014). As it was mentioned earlier, SOD and CAT are antioxidant enzymes that defense cells against the damaging effect of ROS (Ighodaro & Akinloye, 2017).

The product of lipid peroxidation process, MDA, is known as a marker of oxidative damage (Nagamani *et al*., 2015). The results of antioxidant activity assay in gastric tissue (Table 4.5.) showed significant antioxidant response from 2-pentadecanone toward free radicals due to a rise in the level of SOD and CAT and decline in MDA level, in comparison to negative control group, suggested the potential antioxidant effect of this compound in ethanol-induced ulceration in rats.

Further protective role of 2-pentadecanone was determined by detecting NO level in gastric tissue. NO has a significant function in the host defense and inflammatory response, and also exert a protective role in ethanol induced gastric mucosal injury (Pan *et al*., 2005). NO was reported as a protective molecule against gastric ulcer in another study too, where its protective effect was suggested by supporting gastric mucosal blood flow, mucus secretion and diminishing inflammation (Sistani Karampour *et al*., 2019).

Hence, reduction in the level of NO, support development of hemorrhagic lesions due to low blood flows. Reduced NO level triggers a great flow of K^+ and Na^+ and pepsin secretion associated with higher H^+ release. NO is reported to provide a defensive response via blocking neutrophils infiltration and inflammatory mediators followed by the suppression of ethanol induced gastric damage (Moghadamtousi *et al*., 2014). Supportive effect of NO was also reported in a recent study which, has emphasized on the protective effect of NO against gastric ulcer by supporting gastric mucosal blood flow, mucus secretion and diminishing inflammation (Sistani Karampour *et al*., 2019). Hence, we assumed that any bioactive potential agent with capability of inducing NO generation offers a protective action against ethanol induced gastric mucosal injury. Thus, in this study, 2-pentadecanone was tested for its potential to stimulate NO production. As a result, this compound showed its positive response at both doses (10 and 20 mg/kg) in comparison to negative control group where absolute ethanol could significantly inhibit production of NO (Table. 4.5).

Further investigation on the gastro-protective effect of 2-pentadecanone was done under histology analysis. As was detected in the gross morphology observation of stomach tissues, histological assessment of the stomach tissue revealed specific characterizations (Figure 4.11 and 4.12). Severe mucosal injury, submucosal edema and inflammatory infiltrations were detected as a result of absolute ethanol administration (Figure 4.11 A). In addition, histology sections of pre-treated groups displayed that oral administration of 20 mg/kg omeprazole and 2-pentadecanone (10 and 20 mg/kg) conspicuously defended the gastric tissue from hemorrhagic ulceration with less mucosal infiltration and submucosal edema. Although, 2-pentadecanone showed its promising effect in both doses, oral administration of 20 mg/kg of this compound illustrated more similar histology characterization to positive control group, where

newly formed submucosal layer and mucosal integrity is developed (Figure 4.11 B and D).

Mucins are carbohydrates found in epithelial cells of the gastrointestinal tract. Their function include selective permeability to gastric acid, antibacterial activity, lubrication of the luminal contents, regulation of epithelial dehydration, inhibition of tissue edema and suppression of tissue metastasis (Prasanna, 2016). The luminal surface of stomach is masked with a protective mucus layer. Mucus is formed of a complex mixture in which the major constituent is mucin followed by water, electrolytes, sloughed off cells, enzymes and some other materials including bacteria (Ichikawa & Ishihar, 2011).

In this study glycogen content of gastric mucosa was detected via PAS staining and the positive result appeared in magenta color (Moghadamtousi et al., 2014). Microscopic detection of PAS staining showed intense magenta color in all groups except negative control group, which resulted in depletion of glycogen content in this group (Figure 4.12). However, the PAS staining intensity was increased more in pre-treated group with 20 mg/kg 2-pentadecanone compare to low dose treated group (Figure 4.12 C and D), which approximates the influence of standard drug (omeprazole). The microscopic findings of PAS staining have suggested the potential effect of 2-pentadecanone at both doses and slightly higher in pre-treated group with 20 mg/kg, in the induction of higher mucus secretion.

Finally, gastro-protective effect of 2-pentadecanone was confirmed by detecting the expression intensity of Hsp70 and Bax protein in stomach tissue. Hsp70, which has a vital responsibility in the posttranslational modifications of polypeptides, was considered as a protective factor against gastric ulcerations (Moghadamtousi et al.,

2014; Suemasu et al., 2009; AL-Wajeeh et al., 2017). The microscopic results of the current study revealed that mucosal expression of Hsp70 protein was up-regulated in pre-treated groups with 2-pentadecanone (10 and 20 mg/kg) and omeprazole (Figure 4.14). To confirm the up-regulation of Hsp70 protein, expression percentage of this protein was detected based on the brown color intensity analyzed by Image J in each group (Figure 4.15).

The study conducted by Suemasu et al claimed that Hsp70 protect gastric mucosa from injury by suppressing apoptosis (Suemasu et al., 2009). Bax protein is a key factor that stimulates mitochondrial function to initiate apoptosis process; hence suppression of Bax protein expression diminishes cellular destruction and stomach tissue injury (Moghadamtousi et al., 2014). Thus, in this study we have examined the expression of Bax protein in the stomach tissue. As it is shown in Figure 4.14, the expression of Bax protein is higher in negative control group (brown color), while the expression of this protein was significantly reduced in pre-treated groups with omeprazole and 2-pentadecanone (10 and 20 mg/kg). The higher Bax protein expression (brown color) in negative control group indicates the presence of cellular destruction and stomach tissue injury, while its reduction in groups pre-treated with omeprazole and 2-pentadecanone at both doses showed inhibitory effect of these drugs against stomach tissue injury.

After obtaining promising findings extracted from the effect of 2-pentadecanone, this compound cannot go beyond the discovery stage yet, as drug discovery is a complex process. Drug discovery is an intensive and lengthy task, which requires huge investments by pharmaceutical industry with the aid of government. For a potential therapeutic agent to find its way to the market, it must go through series of successful phases of clinical trials, and meet new drug approval process, called New Drug

Application in the United States. There are challenges associated with this process, for example; animal model is not enough to recapitulate an entire disease, or the unknown physiological process associated with the drug efficacy on biological markers. Moreover, there is a high degree of uncertainty that the potential drug will actually succeed (Sheena et al., 2012).

In fact, 92% of successful drugs in animal study model fail in clinical trials, however, this high percentage has never concluded the ineffectiveness of animal studies. Indeed, animal studies are not expected to be completely predictive as human trials are not as well, since it is not until the potential drug is made available to the public that side effects are seen. However, the question that why and where compounds fail in preclinical studies (animal studies) remain a significant remark. Insights into this question leads to the focus on the physiochemical properties of the compounds, where a link between the physiochemical characteristics of the compounds and human trial failure was suggested due to safety issue. This property suggested, some tested compounds represent safety profile in animal models while showing toxicity in clinical studies. Generally, reasons for the failure of candidate compounds to become drugs were classified as follow: 40% failure due to pre-clinical testing, 32% failure due to developer rationalized portfolio, 8% failure due to clinical safety, 7% failure due to 86 commercial reasons, 5% failure due to pharmacokinetics, 5% failure due to other factors, and 4% failure due to poor efficacy (Magee, 2019).

CHAPTER 6: CONCLUSION

In conclusion, the results of this study showed positive effect of 2-pentadecanone on both, skin wound healing process and inhibition of ethanol induced mucosal ulceration in rats. The promising effect of 2-pentadecanone was determined based on its antioxidant activity, diminishing inflammation, up regulating Hsp70 and down regulating Bax protein in both skin and stomach tissues, and supporting collagen synthesis in skin tissue in addition to increasing mucus production in stomach tissue.

6.1 Limitations of the study

Referring to the limitations of this study, the limited number of rats restricted us from having a bigger samples size for greater confirmation of the results. In addition, the limited number of rats prevented us from testing 2-pentadecanone for its side effects and compares it to the side effects of omeprazole to ensure this compound can minimize the negative impacts of omeprazole. Little number of prior research studies on the pharmacological effect of 2-pentadecanone limited us from having a wider view on the activity of this compound. Moreover, the narrow funding source of this study restricted us to the detection of certain proteins in tissue sample.

6.2 Future recommendation

Although 2-pentadecanone showed promising effect in this research, however as a future recommendation, suitable encapsulation of 2-pentadecanone with pH tolerance can be considered to enhance anti-ulcer drug efficacy in stomach, and for skin wound healing an enhanced formulated based cream can be considered. In addition, 2-pentadecanone can be tested for its long-term side effects.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of publications

1. Kamran, S., Karimian, H., Salehen, N., Khalajhedayati, A., Razak, B. A., & Majid, N. A. (2019). Acute toxicity and gastroprotective effect of 2-pentadecanone in ethanol-induced gastric mucosal ulceration in rats. *Internationa Journal of Pharmacology*, *15*(8), 944-952.

Paper presented

2. Kamran, S., Karimian, H., Salehen, N., Khalajhedayati, A., Razak, B. A., & Majid, N. A. (2019). *Acute toxicity and gastroprotective effect of 2-pentadecanone in ethanol-induced gastric mucosal ulceration in rats*. Paper presented at the Graduate Research Symposium at Taylor's University, 26th November 2019, Kuala Lumpur, Malaysia.