MEDICINAL POTENTIAL OF 3,4,5-TRIHYDROXY-N-O-[(2-METHYL-1H-INDOL-3-YL)-METHYLIDENE] BENZOHYDRAZIDE

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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ABSTRACT

Background: In the new drugs design, investigation of hybrid molecules that might possess a synergistic result that leads to a greater activity than each of its constituents may provide a compound with more enhanced pharmacological properties. Aim: Subsequently a newly synthesized 3,4,5-trihydroxy-N'-[(2-methyl-1H-indol-3-yl)-methylidene]benzohydrazide (TIBH) was tested for wound healing, ulcer prevention antioxidant, cytotoxicity and anti-microbial activities. Materials and methods: 1, 1-diphenyl-2-picryl hydrazyl (DPPH) and Ferric-reducing antioxidant power (FRAP) assays were used to test TIBH antioxidant activity. 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay was carried out to measure the TIBH cytotoxicity against human normal fibroblast. Minimal inhibitory concentration (MIC) method was performed to test TIBH antimicrobial effect against two standard strains of *Staphylococcus aureus* and *Escherichia coli* which are known pathogens to cause wound infections. Gastro-protective and wound healing activity of TIBH were studied *in vivo*. Results: TIBH showed a remarkable DPPH radical scavenging activity, with IC_{50} values of 33.8 µg/ml and a very strong FRAP value when compared to reference controls. Cytotoxicity result showed TIBH in concentration more than 154 µM (50 µg/ml) was toxic against human normal fibroblast. Antimicrobial assay showed TIBH MIC against two tested strains was recorded greater than the highest tested concentration of the TIBH (200 µg/ml). Evaluation of the effect of TIBH in wound healing showed that TIBH 10 mg/kg increased wound closure, promoted faster reepithelization and more collagen synthesis, decreased number of inflammatory cells, decreased Bax protein expression and finally increased SOD activity and decreased lipid peroxidation. In ulcer prevention study TIBH increased mucus secretion, decreased

gastric acidity, up-regulation of HSP70 protein, down-regulation of Bax protein, decrease of the lipid peroxidation and the increase of the SOD activity in gastric tissue. Acute toxicity assay exposed valuable information on the safety of this compound. Conclusion: In conclusion TIBH showed to be a strong antioxidant, a wound healing and ulcer preventer promoter.

ABSTRAK

Latar belakang: Apabila mereka-bentuk ubatan baru, penyiasatan molekul hibrid yang mungkin memiliki sinergi boleh membawa kepada peningkatan aktiviti farmakologi yang lebih besar berbanding setiap juzuk individu. Matlamat: Kompaun 3,4,5-trihydroxy-N'-[(2-methyl-1H-indol-3-yl)-methylidene]benzohydrazide (TIBH) yang baru disintesis diuji untuk menyembuhkan luka, antioksidan mencegah ulser, kesan sitotoksik dan aktiviti anti-mikrob. Metodologi dan bahan-bahan: Ujian DPPH dan FRAP digunakan untuk menilai aktiviti antioksidan TIBH. Ujian MTT dilakukan untuk menyukat kesan sitotoksik TIBH ke atas sel fibroblas manusia normal. Kaedah MIC dilakukan untuk menguji aktiviti antimikrobial TIBH ke atas dua jenis bakteria *Staphylococcus aureus* dan *Escherichia coli* standard yang diketahui adalah patogen penyebab jangkitan luka. Perlindungan gastrik dan aktiviti menyembuh luka TIBH dikaji secara *in vivo*. Keputusan: TIBH menunjukkan DPPH aktiviti memerangkap radikal yang baik, dengan nilai IC_{50} sebanyak 33.8 μg/ml dan nilai FRAP yang sangat tinggi berbanding dengan kawalan rujukan. Keputusan kesan sitotoksik menunjukkan TIBH yang berkepekatan lebih daripada 154 μM (50 μg/ml) adalah toksik terhadap sel fibroblas manusia normal. Ujian antimikrobial menunjukkan MIB TIBH terhadap dua strain teruji direkodkan lebih besar daripada kepekatan teruji tertinggi TIBH (200 μg / ml). Penilaian kesan TIBH untuk menyembuhkan luka menunjukkan bahawa 10 mg/kg TIBH meningkatkan liputan luka, menggalakkan penumbuhan semula epitelium dengan lebih cepat dan sintesis kolagen, mengurangkan bilangan sel radang, mengurangkan ekspresi protein Bax, meningkatkan aktiviti SOD, dan mengurangkan pengoksidaan lipid. Di dalam kajian pencegahan ulser, TIBH menambahkan rembesan mukus, mengurangkan keasidan gastrik, meningkatkan protein HSP70, menurunkan protein Bax, mengurangkan pengoksidaan lipid, dan meningkatkan aktiviti SOD di dalam tisu gastrik. Ujian toksik akut mengesahkan kompaun ini selamat untuk diguna.

Kesimpulan: TIBH merupakan antioksidan yang kuat, penawar luka yang menggalakkan, dan pencegah ulser gastrik.

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Last but not least, I would like to thank my parents and my brothers, for all their love and encouragement. This work is dedicated to my beloved parents who raised me with love of science, and supported me in all my pursuits.

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LIST OF SYMBOLS AND ABBREVIATIONS

^{o}C	:	Degree Celsius
DAB	:	3,3‟-diaminobenzidine
DMSO	:	Dimethyl sulfoxide
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
FRAP	:	Ferric Reducing Antioxidant Power
H&E	:	Haematoxylin and eosin
HRP	:	Horseradish peroxidase
Kg	:	Kilogram
M	:	Molar (g/L)
MDA	:	Malondialdehyde
mg	:	Miligram
MIC	:	Minimal inhibitory concentration
Min	:	Minutes
mm	:	Milimeter
MS222	:	Tricaine methanesulfonate
MTT	:	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromid
nm	:	Nanometer
NSAIDs	:	Non-steroidal anti-inflammatory drugs
PBS	:	Phosphate Buffered Saline
ROS	:	Reactive oxygen species
S.E.M	:	Standard error of the mean
SD	:	*Sprogue Dawley* rats
SOD	:	Superoxide dismutase
µl	:	Microliter

µm	:	Micrometer
µM	:	Micromole

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Peptic Ulcer

Peptic ulcer occurs in more than 10% of world population (Ishida et al., 2010). It is one of the most important ailments of gastrointestinal tract in the world and it becomes a global problem due to its increasing morbidity and mortality (Martins et al., 2014). Peptic ulcer defined as a mucosal damage with a length of more than 5 mm which is caused due to the demolition of the balance between defensive and aggressive factors. Aggressive factors are caused by like *Helicobacter pylori*, non-steroidal anti-inflammation drugs (NSID), alcohol consumption, overproduction of acid and pepsin, age-related decline of the prostaglandin level, stress and smoking (Abdelwahab et al., 2013).

Currently, researchers are studying on this common pathology & many drugs are available including prostaglandins, nitric oxide (NO) and hydrogen sulfide (H_2S), Moreover, anti-inflammatory drugs endowed with dual cyclooxygenase inhibitors (Blandizzi et al., 2009), agonists of the histamine H_2 receptor (Ranitidine) and the irreversible proton pump inhibitors (Omeprazole) (Martins et al., 2014). Conversely, sometimes anti-acid drugs are ineffective and weaken the absorption of calcium, iron, magnesium and vitamin B_{12} (Ham & Kaunitz, 2008). Thus, medicinal plants and synthetic compounds that can contribute in ulcer healing and in the prevention of the ulcer reoccurrence have been used as alternative treatments (Abdelwahab et al., 2013). In former studies, numerous synthesized chemical compounds were discovered to have biological properties together with gastric ulcer prevention potential (Abdulla & Abdelwahab, 2012; Gwaram et al., 2012; Hajrezaie et al., 2012; Golbabapour et al., 2013; Dhiyaaldeen et al., 2014; Halabi et al., 2014; Nazarbahjat et al., 2016; Salama et al., 2016).

1.2 Wound Healing

Wound is defined as a disruption of the cellular and anatomic integrity of a tissue and may occur due to physical, chemical, thermal, microbial, or immunological tissue trauma (Leaper & Harding, 1998; Gantwerker & Hom, 2011; Moghadamtousi et al., 2015). Wound healing is a dynamic overlapping process which includes hemostasis, inflammation, proliferation and remodeling. All phases must take place in proper order and time frame to heal the wound successfully. Hemostasis starts with vasoconstriction to stop bleeding. Bleeding on the site of injury will trigger platelets aggregation. Platelets will release clotting factors, growth factors and cytokines. These released factors lead the wound healing to its next step which is inflammation. During this phase the neutrophils travel into the wound area to begin phagocytosis which eliminates debris and bacteria and damaged cells. Neutrophils are soon followed by macrophages. Macrophages continue cleaning of the wound and releasing of factors that progress the healing process. In the following step, proliferation, the wound area is filled up the granulation tissue. Fibroblasts begin to build new extracellular matrix. In this stage angiogenesis and re-epithelization occur. During the remodeling phase new collagen is produced. Wound healing is a complex process of cellular and biochemical interactions involving various cells such as keratinocytes, fibroblasts and endothelial cells (Krishnamoorthy et al., 2012). Many factors affect the wound healing like malnutrition, infection, insufficient oxygenation, stress, smoking alcoholism‟s, age, diabetes and obesity (Gantwerker & Hom, 2011).

Oxygen plays pivotal roles in the wound healing process, such as oxidative bacterial killing, collagen synthesis, angiogenesis, and epithelialization; hence the wound healing process is impaired under hypoxia. Both enzymatic and non-enzymatic antioxidants have a very important role in wound healing. The generated reactive oxygen species (ROS) directly attack invading pathogens, and kill them. Though, extra production of

superoxide harms the surrounding tissues. Superoxide is dismutated to hydrogen peroxide (H_2O_2) and molecular oxygen by either superoxide dismutase (SOD) or a spontaneous reaction. H_2O_2 is detoxified by peroxidases such as catalase and glutathione peroxidase (GPX) to avoid the production of hydroxyl radicals, the most harmful ROS (Kurahashi & Fujii, 2015).

Misuse of antibiotics to prevent bacterial infections resulted in the increase of bacterial resistance. The frequency of drug-resistant bacteria is increasing. Drug-resistant bacteria tend to spread epidemically in hospitals and are involved in producing nosocomial infections (Hospital-acquired infections). Gram positives *Staphylococcus aureus* and Gram negatives *Escherichia coli* are among the highest abundant species were detected to infect the wounds (Petkovšek et al., 2009; Rhoads et al., 2012).

Wound and tissue linked diseases are the most known and damaging sorts of trauma around the world and thousands are still passing away each year due to the lack of adequate treatment. When wound fail to heal in a timely and orderly manner, resulting in chronic, non-healing wounds that require continued management. It is estimated that over $25 billion is spent each year on the treatment of chronic wounds alone (Sen et al., 2009). Wounds affect well-being, working capacity and independence of individuals (Maver et al., 2015). Currently various techniques and strategies are being used in for wound healing. In brief the approaches include the use of autografts, allografts, cultured epithelial autografts, delivery of growth factors or siRNA, targeting microRNA, stem cell therapy, use of the sensors to monitor and manage of the wound microenvironment, use of wound dressings based on biocompatible and biodegradable polymers, marine-derived biological macromolecules, plant extracts and synthetic compounds (Boateng et al., 2008; Chandika et al., 2015; Dreifke et al., 2015; Maver et al., 2015). The drug supply through the skin has long been an excellent root because of simplicity,

availability of vast surface area, vast exposure to the circulatory and lymphatic networks, and its noninvasive nature (Geethalakshmi et al., 2013). The diversity of wound natures has resulted in an extensive range of wound dressings with new products commonly presented to target various aspects of the wound healing process. The perfect dressing should succeed in rapid healing. In this study the wound healing potential of a newly synthesized compound was evaluated as a wound dressing material. Despite recent efforts, the current wound therapy methods are often painful and inadequate which highlights the need of development of effective wound therapy methods, products and drugs (Uzun et al., 2013).

1.3 The Compound

Indole derivatives are biologically essential chemicals with a wide range of therapeutic potentials. They possess antibacterial, antifungal, antiviral, antimalarial, anti-HIV, anticancer, and antioxidant properties. Indole compounds are very efficient antioxidants, keeping both lipids and proteins from peroxidation, and it is identified that the indole structure effects the antioxidant efficacy in biological organism (Khaledi et al., 2011). Gallic acid on the other hand is a polyphenol with much biological and pharmacological potential. Polyphenols form a key class of chemo-preventive agents because they can prevent the generation of ROS (Guziket al., 2010). In the design of new drugs, hybrid molecules may have a synergistic effect, resulting in a higher potential than each of its components which may lead to compounds with interesting pharmacological properties.

3,4,5-trihydroxy-N'-[(5-methyl-1H-indol-3-yl)-methylidene]benzohydrazide (TIBH) was subsequently synthesized upon the reaction of the appropriate indole carboxaldehydes with gallic hydrazide. This compound was found to exhibit significant

DPPH radical scavenging potential and also possessed inhibitory effect against lipid peroxidation (Khaledi et al., 2011).

The current study was carried out to determine wound healing, ulcer prevention, acute toxicity, antioxidant, cytotoxicity and anti-microbial effects of TIBH.

1.4 Research Objectives

1.4.1 General objective

Evaluation of the wound healing activity of TIBH (*In vivo* and *in vitro)*

Evaluation of the ulcer prevention activity of TIBH *(In vivo* and *in vitro)*

1.4.2 Specific objectives

Evaluation of the acute toxicity of TIBH *(In vivo)*

Evaluation of the cytotoxicity of TIBH *(In vitro)*

Evaluation of the anti-oxidant activity of TIBH *(In vitro)*

Evaluation of the anti-microbial activity of TIBH *(In vitro)*

CHAPTER 2: LITERATURE REVIEW

2.1 Peptic Ulcer Defensive Factors

2.1.1 Mucus-Bicarbonate Barrier

Mucus-bicarbonate-phospholipid barrier is the first line of gastric mucosal defense mechanism (Lichtenberger, 1999). The gastric mucosa contains the glands and the gastric pits and its cover consists of mucus gel, bicarbonate anions and phospholipids. The mucus gel is secreted by surface epithelium that prevents enzymatic digestion of epithelium through the inhibition of pepsin penetration. It is about 95% water with various mucin glycoproteins. Prostaglandins, acetylcholine, gastrin, secretin and other gastrointestinal hormones regulate the mucus production (Allen & Flemström, 2005). Bicarbonate ions preserves the pH near to 7 at the epithelial surface representing another first type of defense against stomach acid. Secretion of bicarbonate from the apical surface epithelial cells is regulated by a Cl-/HCO3- anion exchanger. Several factors regulates bicarbonate production like prostaglandins, acid, melatonin, uroguanylin corticotrophin-releasing factor and orexin A (Fornai et al., 2011). In case of mucus-bicarbonate barrier abruption by aggressive factors, second line of defense including acid neutralizing agents, epithelial repair or mucosal blood flow initiates its role.

2.1.2 Epithelial Cells

The next line of mucosal defense are the continuous, hydrophobic cells of surface epithelium. This layer of cells secret mucus, bicarbonate and some of the other constituents of the mucosal defense mechanism. Due to the existence of phospholipids on their surface, epithelial cells are hydrophobic and are consequently able to resist against acid- and water-soluble damaging agents (Lichtenberger, 1999). To form an

unbroken barrier to avoid back diffusion of pepsin and acid, epithelial cells are strictly linked by tight junctions (Allen & Flemström, 2005). Colonization of bacteria are prevented at the mucosal surface by the action of two cationic peptides: Cathelicidin and beta-defensin (Yang et al., 2006). Heat shock proteins are additional important defensive factor is presented in the epithelial cells (Tanaka et al., 2007).

2.1.2.1 HSP70

HSP70 protein is expressed in mammalian cells and belongs to the family HSP proteins. It protects the cells by decreasing intracellular protein denaturation induced by oxidative stress or heat shock. This 70 kDa protein is a highly conserved and richly formed protein in the occurrence various forms of stress like temperature increments, toxic substances, infection and proliferation. These proteins can prevent protein denaturation and protect cells against injury (Tanaka et al., 2007). Various studies have shown that overexpression of HSP70 protein that is prompted by ulcer prevention chemicals, plant extracts or antiulcer drugs, can improve the gastric ulcers or prevents its occurrence. HSP70 protein increases the mucosal blood flow and decreases the intracellular protein denaturation. However, the exact protective mechanisms of this protein are yet not known (Shichijo et al., 2003; Moghadamtousi et al., 2014; Rouhollahi et al., 2014).

2.1.3 Mucosal Cell Renewal

Continuous proliferation of mucosal epithelial progenitor cells is maintained to preserve the integrity and renewal of injured or old gastric epithelium. It takes months and a week to respectively renew the glandular and epithelial cells. On the other hand,

the injured surface epithelium replacement takes place in only a few minutes and it happens due to the migration of the preserved cells to the damaged area (Laine et al., 2008). Many factors are involved in the process of mucosal cell renewal including overexpression of epidermal growth factor receptor (EGF-R), transforming growth factor (TGF), insulin-like growth factor-1 (Nguyen et al., 2007). In addition, presence of epithelia growth factor (EGF) in stomach juice stimulates mucosal cell proliferation (Milani & Calabrò, 2001). Gastrin and Prostaglandin E2 (PGE2) induce mucosal cell renewal by stimulation of mitogen-activated protein kinase (MAPK) mechanism (Pai et al., 2002). Expressed survivin by mucosal progenitor cells acts as an anti-apoptotic protein and prevents the apoptosis of the mucosal progenitor cells (Chiou et al., 2005).

2.1.4 Mucosal Blood Flow

Mucosal blood flow carries oxygen and nutrients to the gastric mucosa and removes the noxious agents from the gastric mucosa. It efficiently dilutes acid back diffusion and prevents the gastric tissue necrosis. The mucosal blood flow is increased by NO and prostacyclin (PGI2) release which act as vasodilators. NO release prevents the detrimental effects of endothelin 1, leukotriene C4, thromboxane A2 and ethanol. NO synthase inhibition increased mucosal damages (Fornai et al., 2011). H_2S, as another agent, prevents the formed damages of the gastric mucosal by NSAID. H_2S can protect the gastric mucosal against injury. It decreases the tumor necrosis factor (TNF) expression and diminishes the leukocyte bond to vascular endothelium (Fiorucci et al., 2006).

2.1.5 Prostaglandins

Short half-life (seconds to minutes), 20-carbon fatty acids of prostaglandins are generated from arachidonic acid under the action of cyclooxygenase enzyme. prostaglandins control various of the constituents of mucosal defense mechanisms including: increase of the mucosal blood flow, induction of mucus and bicarbonate secretion, acid production prevention, recruitment of leukocytes into the mucosa, inhibition of tumor necrosis factor (TNF)-a and interleukin (IL)-1 release. NSAIDs exerts their harmful effects on the stomach via the inhibition of the prostaglandin synthesis (Martin & Wallace, 2006).

2.1.6 Neuro-Hormonal Defense

Central nervous system and hormones are two important mechanism of Gastric mucosal defense (Laine et al., 2008). Mucus secretion, increases of intracellular pH, endocrine responses to stress, inhibition of gastric emptying and motility are some of the activities are resulted by central vagal stimulation (Fornai et al., 2011). Neurokinin A, thyrotropin-releasing hormone, cholecystokinin, including gastrin-17, peptide YY and bombesin, EGF are some of the other hormones regulate the gastric protective mechanism (Mózsik et al., 2001; Peskar, 2001). Glucocorticoids on the other hand induced their stress respond gastro-protective activity via increase of the mucosal blood flow, maintenance of the blood glucose level, increase of the mucus secretion and inhibition of microvascular permeability and gastric motility (Filaretova et al., 2007).

2.2 Peptic Ulcer Aggressive Factors

2.2.1 *Helicobacter Pylori*

H. pylori for the first time was detected in many patients with peptic ulcer in 1983. Before that it was presumed that excessive gastric acid secretion was the cause of peptic ulcer. More than half of the world‟s people is assumed to be infected with this bacteria yet, 5 to 10% of them generate peptic ulcer. The reason is the variation in the pathogenicity of various strains of this bacteria. *H. pylori* is the main cause of peptic ulcer. Induction of local injury by bacteria leads to inflammation and epithelial damage. Under the action of urease enzyme in this bacteria, urea is converted to carbon dioxide (CO_2) and ammonia (NH_3) which results in the generation of an alkaline environment in stomach. In response to this alkaline environment, D cells are inhibited from somatostatin production. Inhibition of somatostatin prevents the blockage of G cells from gastrin secretion. Thus, gastrin secretion increases and its effect on the parietal cells leads to the increase of the acid secretion. Moreover, the bacteria interrupt the activity of the responsible neurons for gastric acid secretion (Proctor & Deans, 2014).

2.2.2 NSAIDs

Almost 1-2% of NSAIDs consumers will be affected by peptic ulcer (Sostres et al., 2010). NSAIDs have various mechanism to induce ulcer. The injury is caused by acetylsalicylic acid (ASA) for example, is systemic and local. Acidic NSAIDs like diclofenac and aspirin have cytotoxic and proliferation inhibitory effects on mucosal epithelial cells. However most of NSAIDs inhibit cyclooxygenase and reduce prostaglandin. Prostaglandins roles in gastro-protection is explained in section 2.1.5 (Proctor & Deans, 2014).

2.2.3 Alcohol Consumption

The effects of ethanol on the gastric mucus membrane are damaging and dose-dependent with higher concentrations being more harmful. The injury occurs 30 minutes after the intake and is maximized at about 60 minutes later (Stermer, 2002). Some previous studies showed that alcohol concentration below 5% increases acid secretion mainly by exciting the secretion of gastrin and to a lesser extent by a straight influence on the parietal cells. Reversely, an alcohol concentration of higher than 5% has been showed to have no effect on gastric acid production (Chari et al., 1993; Singer et al., 1987; Stermer, 2002). Alcohol increases the haemorrhagic ulcer, gastric submucosal oedema and infiltrated leucocytes (Nordin et al., 2014). Alcohol induced gastric irritations might occur due to the mucosal vasodilation as a result of endothelin-1 release into the blood. Additionally ethanol produce apoptosis that exerts cell death (Gulia & Choudhary) 2011. Alcohol also destroys the blood vessel resulting in the increase of vascular permeability, the edema formation and the epithelial cell loss.

2.3 Current Wound Healing Approach

2.3.1 Donor Keratinocytes

Cells control their own growth in addition to the growth of nearby cells. Hereby, they employ various cell signaling chemicals. Keratinocytes have an effective role in wound healing through the creation of platelet clots, the release of cytokines and numerous growth factors. However this ability of keratinocytes reduces with the age of donor as proliferation of keratinocytes reduces in adult donor (Dreifke et al., 2015; Guerid et al., 2013).

2.3.2 Graft

In split-thickness autograft, the sheet of connective tissue beneath the skin of the donor site (fascia) is removed and grafted to the recipient site of injury. Split-thickness autograft exerts a high healing rate and an enhanced cosmetic results. However, pain, possibility of the infection, limitation of the number of donors and scarring are some of its disadvantages (Wainwright, 1995; Dreifke et al., 2015). Difficulties associated with allogeneic skin grafts gave the need for use of autologous grafts. In cultured epithelial autografts, epithelial cells are attained with punch biopsy. They are subsequently expanded with the help of epithelial growth factor (EGF) and cholera toxin *in vitro*. Donor cells can be increased to one thousand fold in one month of culture. Cultured epithelial cells are detached by enzyme dispase and are grafted onto the wound site. This method is firstly done by (O'Connor et al., 1981). Cultured autologous grafts reduce the threat of graft rejection. Cultured epithelial autografts is a very potential treatment compared to other conventional treatments. However, the graft needs to be attached to the wound bed membrane to proliferate and differentiate. Thus this stage is one of the success rate determinative of this method. Thin layer of graft put it at the risk of being digested by the enzymes within the recipient wound tissue. Also the required time to culture the epithelial cells sheet reduce from the value of this method. Graft contraction on the other hand leads to a poor cosmetic outcomes. The use of graft in wound therapy to a great extent depends on the age and health condition of the donor. Problems associated with the use of grafts highlights the need of development of other effective wound therapy methods (Dreifke et al., 2015).

2.3.3 Wound Dressings

Various types of wound dressings in the market target different aspects of the wound healing including the wound hydration preservation to improve regeneration, the prevention of infection, the inhibition of wound base disruption, provision of thermal insulation, absorption of blood and excess exudate and the provision of wound healing accelerators like vitamins, minerals and growth factors. In general, a perfect dressing should increase the rate of the wound healing with a reasonable charge and also with the least of inconvenience for the patient. The most currently presented novel polymers used for the delivery of bioactive agents to the wound site are chitosan, hyaluronic acid, collagen and silicon. Different type of wounds need different types dressings. In fact, no single dressing is appropriate for the management of all types of wounds. Additionally, the wound healing mechanism has various stages that can be targeted by various dressing (Boatenget al., 2008).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

Intrasite gel (a trademark for Smith and Nephew Healthcare Limited) was purchased from the Experimental Animal House Unit, Faculty of Medicine of University of Malaya. Intrasite gel is a colorless transparent aqueous gel, which contains 2.3% of a modified carboxymethylcellulose (CMC) polymer together with propylene glycol (20%) as a humectants and preservative. When topically applied on the wound, Intrasite gel absorbs the excess secretions and generates a moist condition at the wound without causing tissue maceration. It does not harm viable tissue or the skin around the wound and is non-adherent (Williams, 1994). Omeprazole, as an anti-ulcer medicine and positive control was purchased from the University of Malaya, Medical Center. Omeprazole is a proton pump inhibitor drug that has being employed for the treatment of peptic ulcer ailment. It acts as enzymes inhibitor and stops the stomach from producing excessive acid that can harm the gastric wall. Omeprazole was dissolved in 1% w/v CMC and administered orally to the rats in a dosage of 20 mg/kg body weight (5 mL/kg) (Wasman et al., 2011). The TIBH was synthesized by chemistry department, University of Malaya. Microanalyses were carried out on a Perkin-Elmer 2400 elemental analyzer. 1H-NMR and 13C-NMR spectra were determined with a Lambda JEOL 400 MHz FT-NMR (1H-NMR: 400 MHz and 13C-NMR: 100.4 MHz) spectrometer. Figure 3.1 illustrates the structural formula of the TIBH (Khaledi et al., 2011). Dimethyl sulfoxide (DMSO) (Sigma Aldrich, Germany) is an organosulfur compound and an important solvent that dissolves both polar and nonpolar compounds and is miscible in an extensive range of organic solvents as well as water (Brayton, 1986). It was used to solubilize the TIBH. Carboxymethyl cellulose (CMC) and Glycerol (Sigma Aldrich, Germany) were each separately used as a dressing vehicle (Stout & McKessor, 2012; Dhiyaaldeen et al., 2014). Here, combination of the two were

used as TIBH vehicles respectively in concentration of 2% and 20%. Ketamine and xylazine were used to anesthetize and sacrifice of the animals (Sigma Aldrich, Germany). SOD and MDA commercial kits were purchased from the Cayman Chemical Company (Cayman, USA).

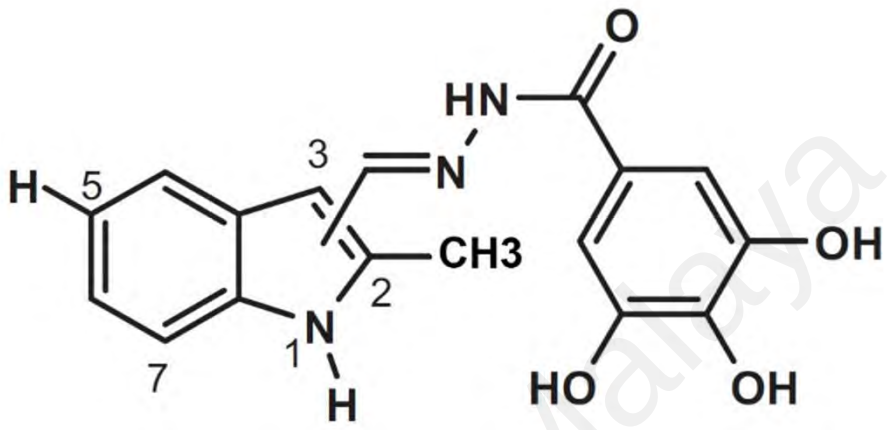

Figure 3.1: Structural formula of the 3,4,5-Trihydroxy-N 0-[(2-methyl-1H-indol-3-yl)-methylidene] benzohydrazide (TIBH)

3.2 Methods

3.2.1 Evaluation of Antioxidant Activity

3.2.1.1 1, 1- diphenyl-2-picryl hydrazyl (DPPH) measurement

Free radical scavenging activity of TIBH various concentrations were measured by 1, 1- diphenyl-2-picryl hydrazyl (DPPH) assay as described by (Osorio et al., 2012) with some modifications to be adjusted for 96-well plates. This method was theorized by (Blois, 1958). Briefly, 0.1 mM solution of DPPH in ethanol was prepared. A total of 190 µl of DPPH solution (0.1 ml) was added to 10 µl of TIBH various concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 µg/ml) in DMSO. DMSO was used as blank. The mixture was shaken vigorously and left at room temperature for 30 min. DPPH solution has a deep violet color and its highest absorbance is at 517nm. The decrease in the reaction mixture absorbance indicated the higher free radical scavenging

activity and was measured at 517 nm by using spectrophotometer. Quercetin and ascorbic acid were used as positive controls and each tests were done in triplicate. The concentration of TIBH required to inhibit 50% of the DPPH free radical (IC_{50}) was obtained using the TIBH dose dependent inhibition curve. The DPPH radical scavenging activity of each concentration was measured according to the following equation: DPPH scavenging effect (%) or Percent inhibition = [(blank absorbance − sample absorbance) / blank absorbance] × 100.

3.2.1.2 Ferric-reducing antioxidant power (FRAP) measurement

This method was performed as described by Benzie and Strain (Benzie & Strain, 1996). It was used to measure the antioxidant potential of the compound for reducing colorless ferric complex (Fe3+ tripyridyltriazine) to a blue color ferrous complex (Fe2+ tripyridyltriazine) solution which absorbs the light maximum at 593nm. The FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mmol TPTZ solution in 40 mmol HCl and 20 mmol iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagents were prepared as follows: 300 mmol/ L acetate buffer, pH 3.6 (prepared by dissolving 3.1 g sodium acetate trihydrate and 16 ml glacial acetic acid up to 1.0 L distilled water); 10 mmol/L TPTZ dissolved in 40 mmol/L HCl; and 20 mmol/L FeCl3.6H2O solution in distilled water. The FRAP reagent was freshly prepared by mixing 25 ml acetate buffer with 2.5 mL TPTZ solution and 2.5 mL FeCl3.6H2O solution. The FRP reagent was prepared fresh and was warmed to 37 C^o in a water bath before use. Quercetin and ascorbic acid were used as positive controls. TIBH, quercetin and ascorbic acid were serially diluted in DMSO from 100 µg/ml to 0.78125 µg/ml. A total of 10 µl of each sample was added to 300 µl of the FRAP reagent (final dilution 1: 34) in a 96 well plate. The absorbance at 593 nm was read in

the beginning (At_0) and after 4 min (At_4) against a blank reagent (DMSO) using a spectrophotometer. The difference between the absorbance values was calculated for each sample. The blue color formation indicates that the sample has a reductant (antioxidant) potential. Freshly prepared aqueous solutions of known Fe^{+2} concentration (FeSO4.7H2O) were used for calibration. The concentration range over which the absorbance showed linear response (100– 1000 mmol/L) was used to calculate a standard curve (Appendix B). Results were expressed as mM of $FeSO_4$.7H2O / dry weight of the compound. All the measurements were done in triplicate and the mean values were calculated (Benzie & Strain, 1996; Wong et al., 2006).

3.2.2 Culture of Human Normal Fibroblast Cells (BJ-5ta)

Human normal fibroblast (BJ-5ta) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin (5000 units/mL) and streptomycin (5000 µg/mL). It is then incubated at 5% CO_2 at 37°C. Medium was changed every 2 days and cells were sub-cultured (split ratio 1:4) every 4 days by trypsinization (0.25% trypsin/0.02% EDTA) (Felice et al., 2015).

3.2.3 Evaluation of Cytotoxicity

MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was used to measure cytotoxicity of the TIBH against human normal fibroblast (BJ-5ta) as described before with some modifications (vanMeerloo et al., 2011). To obtain non-toxic concentration of DMSO for TIBH solubility, DMSO cytotoxicity against BJ-5ta fibroblast cells was tested using the same method. The reduction of tetrazolium salts is a broadly used efficient method to determine various substances cytotoxicity. The yellow

tetrazolium MTT is reduced by metabolically active cells, by the action of dehydrogenase enzymes to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. Briefly BJ-5ta cells were seeded into 96 plate at a density of 5×103 cells per well and incubated overnight. The cells then were treated with various concentrations 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 µg/ml of the TIBH in DMSO and media. Wells with same range of TIBH concentration without the cells were used as blank. Culture media alone was used as negative control. After 48 hours the wells content were discarded. MTT was dissolve in Dulbecco‟s Phosphate Buffered Saline, pH=7.4 (DPBS) to reach 5 mg/ml in concentration. A total of 10 µl of MTT Solution was add to each well to achieve a final concentration of 0.45 mg/ml. After 4 hours of incubation of the plate in the dark at 37^{o}C, the wells content were discarded and DMSO (as a MTT solvent) was added to the wells. Then the absorbance was read at 590 nm. Graph of cell viability (%) vs. the compound concentration was plotted and the cytotoxicity IC_{50} value of the TIBH against human normal fibroblast was determined.

3.2.4 Evaluation of Antimicrobial Activity

Bactericidal effect of TIBH was tested against two standard strains of *Staphylococcus aureus* (ATCC25923) and *Escherichia coli* (ATCC 25922) which are known pathogens to cause wound infections (Kumara et al., 2015). The test was carried out at Department of Microbiology, Universiti Teknologi MARA (UiTM). TIBH was evaluated in seven concentrations of 200, 100, 50, 25, 15, 0 µg/ml using MIC (Minimal inhibitory concentration) method (Andrews, 2001; Pan ek et al., 2006, Wiegand et al., 2008). The MIC is defined as the lowest concentration of the antimicrobial agent that inhibits bacterial growth. Suspensions of equal to 0.5 McFarland standards (5 × 105

cfu/ml), the recommended final inoculum size for broth dilution, were prepared using pathogenic organisms. Sterile BHI Broth (Brain Heart Infusion Broth), TIBH stock solution of 1000 mg/ml in DMSO and bacterial suspension of 1 × 108 cfu/ml in BIHB were mixed according to table 3.1 (test tubes 1-7). All tests were done in triplicate and 45 glass test tubes (12 x 75mm) were prepared for the two strains.To check the sterility, BHI Broth alone was added to three separate test tubes (test tube 8). After overnight incubation at 37 C^{o}, tubes were observed for growth using DensiCHEK Plus (BIOMERIEUX, INC). The DensiCHEK Plus used to measure the turbidity of the microorganism suspension and to determine the McFarland value generated using a single wavelength of 580 nm. The McFarland value is proportional to microorganism concentrations and the reading range is 0.0 - 4.0 McFarland.

Table 3.1: Preparation of TIBH various concentrations in bacterial inoculation of *Staphylococcus aureus* or *Escherichia coli*

	TIBH in DMSO(1000mg/ml)	**Bacterial suspension in BHI Broth (1 × 10^8 cfu/ml)**	**BHI Broth**	**Final concentration of TIBH**	**Final concentration of bacteria**
Test tube 1	200 µl	5 µl	795 µl	200 µg/ml	5×10^5 cfu/ml
Test tube 2	100 µl	5 µl	895 µl	100 µg/ml	5×10^5 cfu/ml
Test tube 3	50 µl	5 µl	945 µl	50 µg/ml	5×10^5 cfu/ml
Test tube 4	25 µl	5 µl	970 µl	25 µg/ml	5×10^5 cfu/ml
Test tube 5	15 µl	5 µl	980 µl	15 µg/ml	5×10^5 cfu/ml
Test tube 6	5 µl	5 µl	990 µl	5 µg/ml	5×10^5 cfu/ml
Test tube 7 Growth control	0 µl	5 µl	995 µl	0 µg/ml	5×10^5 cfu/ml
Test tube 8 Sterility control	0 µl	0 µl	1000 µl	0 µg/ml	0 cfu/ml

3.2.5 Experimental Animals

Old adult healthy Sprague Dawley rats weighing 180-200 g were purchased from the Experimental Animal House Unit, Faculty of Medicine, University of Malaya. The use of animals in this research study was approved by Ethics committee for Animal experiment of the Faculty of Medicine, University of Malaya, Malaysia (Ethic No. 2015-181201/BMS/R/MAAH) and the study was conducted according to the National Academy of Science‟s Guide for the Care and Use of Laboratory Animals (Health, 1978; Pahari et al., 2010).

3.2.6 Evaluation of Acute Toxicity

Healthy females Sprague Dawley rats (n= 18) were allotted equally between three groups (6 rats/group) and categorized as the vehicle (1% CMC) and 300 and 2000mg/kg of TIBH, respectively. The rodents were fasted overnight (food supply but not water supply) preceding dosing. After feeding of the TIBH the animals were retained under monitoring for 30 minutes, 2, 4, 24, and 48 hours, for any toxicological or clinical signs. After 15 days, blood samples of all animals were taken and the animals were euthanized using an overdose of ketamine and xylazine anesthesia. Hematological evaluation of kidney and liver function parameters were done by Medical Laboratory of the Hospital of University of Malaya. Organs (kidney and liver) were evaluated macroscopically and histologically. Sections of kidney and liver were placed in the cassettes, fixed in 10% buffered formalin, processed by tissue-processing machine (Leica, Germany), paraffin embedded, cut to 5μm sections, stained with H&E dye (Appendix C) and observed under microscope to evaluate the tissue structure (OECD, 1994; Fard et al., 2011).

3.2.7 Evaluation of Ulcer Prevention Activity

3.2.7.1 Pre-treatment and induction of gastric ulcer with HCl/Ethanol

In this study, 36 healthy SD rats were acquired from the Animal House, University of Malaya. The experiment was designed having 6 groups while 6 rats were randomly allotted to each group. The rats were fasted for 24 hours before the oral gavage pre-treatment (food but not water). Water supplement had been removed two hours before oral pre-treatment started. HCl/Ethanol-induced ulcer generation was conducted according to the model that previous described (AlRashdi et al., 2012). Accordingly rats in experimental groups were received 50, 100 and 200 mg/kg of TIBH, normal control group and ulcer control group rats were orally received 5ml/kg of 1% CMC each and rats of positive control group were fed with 20 mg/kg of Omeprazole. After sixty minutes, rats of normal group were orally fed with 5 ml/kg of 1% CMC while 5ml/kg of 150 mM (HCl/absolute ethanol) 40:60 v/v were orally fed to the ulcer control, positive control and experimental groups.

After additional hour, all rodents were sacrificed by overdose of ketamine and xylazine (150 and 15 mg/kg) (Wong et al. 2013). The pyloric and the cardiac ends of the stomach were knotted. The stomach was removed and kept into cold phosphate buffer saline (PBS) for further experiments.

3.2.7.2 Evaluation of gastric juice and mucus

The gastric juice content of rat‟s stomachs were collected and centrifuged at 4000 rpm, 25°C for 10 min. The acidity of the resulted supernatant was measured with a digital pH meter that was titrated with 0.1 N NaOH solution. Then, the glandular portion of the stomach was lightly scrapped by a glass slide and weighed (Nordin et al., 2014; Rouhollahi et al., 2014).

3.2.7.3 Evaluation of stomach morphology

The stomach was cut along its greater curvature, distended over a clean and white background for better visualization and photographed. Ulcers showed as elongated bands of hemorrhagic lesions parallel to the long curve of the stomach. The ulcer area on the gastric mucosa was measured using Image J (1.50i). The inhibition percentage (I %) was determined using the subsequent formula where UA (mm2) was the measured ulcer area (Al Batran et al., 2013).

$$(I\ \%) = [(UA\ control - UA\ treated) / UA\ control] \times 100\%$$

3.2.7.4 Histopathological evaluation of gastric ulcer

Finally the gastric tissue specimens were placed in the cassettes and were fixed in 10% buffered formalin. Later, these were processed by tissue-processing machine (Leica, Germany). Tissue embedding, sectioning and staining were performed for all of the tissue samples. Sections of 5µm was stained with H&E dye to evaluate the tissue structure (Taha et al., 2012). Two dyes, Hematoxylin (basic dye) and Eosin (acidic dye) were used for the detection of the nucleus and the cytoplasmic inclusions, respectively (Bancroft & Gamble 2008). Mucosal glycoproteins content of the tissues were evaluated by using of Periodic acid-Schiff (PAS) stain (Appendix D). The sections of the 5µm thick glandular portion of the rat stomach was stained with Periodic acid-Schiff (Moghadamtousi et al., 2014).

3.2.7.5 Evaluation of Bax and HSP70 rexpression in gastric tissues using Immunostaining

Tissue sections were heated at 60^{o}C in a hot-air oven for 25 min (Venticell, MMM, Einrichtungen, Germany). The 5 µm gastric tissue sections were washed with xyleneto remove the paraffin. They were then rehydrated by graded ethanol. Antigen recovery process was done using 10 mM of boiled sodium citrate buffer. Immunostaining was accomplished consistent with manufacturer‟s protocol (Dakocytomation, USA). Concisely, peroxidase block (0.03% hydrogen peroxide containing sodium azide) was used to block the endogenous peroxidase. Washing buffer was used to gently rinse the samples of tissue sections. After that the samples were incubated with HSP70 (1:500) or Bax (1:200) biotinylated primary antibodies for 15 min period. The samples were softly washed with wash buffer and transferred into the buffer bath and then to a humidified chamber. Sufficient amount of Streptavidin–HRP (Streptavidin conjugated to horseradish peroxidase in PBS) was incubated with the samples for 15 min period. Subsequently, the samples again were delicately rinsed in the washing buffer and were transferred to the buffer bath. The samples were incubated with DAB-substrate-chromagen for 5 min. They were then washed and counterstained with hematoxylin for 5 seconds. The samples were immersed 10 times in 0.037 M/L Ammonia and were washed with pure water earlier to the mounting of cover slips. Under a light microscope, positive results of the immunostaining were displayed in brown color (Ismail et al., 2012).

3.2.7.6 Tissue homogenization

The obtained tissues were sliced into sections of nearly 200 mg. One gram of tissue in 8 ml of phosphate-buffered saline (PBS) was homogenized using a Teflon

homogenizer (Polytron, Heidolph RZR 1, Germany). The mixture then was centrifuged at 4,500 rpm for 15 min at 4^{o}C. The resulted supernatant was divided into aliquots and retained at -80^{o}C and then used for the lipid peroxidation (MDA) and SOD enzymatic assay using commercial kits. All processes were preceded in accordance to the manufacture‟s instruction.

3.2.7.7 Evaluation of Lipid peroxidation

Lipid peroxidation is the degradation of lipids happens due to the cellular injuries. It is an indicator of oxidative stress. Malondialdehyde (MDA) as a lipid peroxidation end product of the polyunsaturated lipids of tissues was determined using a Cayman‟s TBARS assay kit.

In brief, 100 µl supernatant of homogenized normal control, ulcer control, TIBH pre-treated and positive control gastric tissues were separately mixed with 100 µl of SDS solution and 4 mL of the colour reagent then boiled (for 1 hour). After that the reaction was immediately ended by transferring the mixture into the ice bath (for 10 min). The mixture was centrifuged for 10 min at 1,600 g at 4^{o}C and left for 30 min. A total of 150µl of the mixture was added to the 96 well plate in triplicate and the absorbance was read at 532 nm. MDA standard curve was plotted and the MDA value of each sample were calculated from standard curve.

3.2.7.8 Evaluation of superoxide dismutase (SOD) activity

Superoxide Dismutase is a mettalloenzyme catalyze the deactivation of the superoxide onions (-O_2) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) so it possesses a very vital function in the system of antioxidant defense. Activity of the

superoxide dismutase (SOD) in the supernatant of all the gastric tissue homogenates was measured using a Cayman‟s assay kit as stated by the kit‟s instructions.

For SOD assay, SOD samples plus with the SOD standard were set up in the assay plate. Briefly, 10 µL of assay standard and 10 µL of the supernatant of normal control, ulcer control, positive control, TIBH pre-treated rats gastric tissue homogenate were separately mixed with 200 µL of diluted radical detector in the wells. Then 20 µL of diluted xanthine oxidase was added to the all wells. After 30 min incubation on the shaker, the absorbance was measured at 440–460 nm.

3.2.8 Evaluation of Wound Healing Activity

3.2.8.1 Induction of excisional wounds

The animals were anaesthetized with 50 mg/kg of ketamine (100mg/mL) and 5 mg/kg of Xylazin (100mg/mL) given intramuscular injection prior to creation of the wounds. The skin was shaved and disinfected with 70% alcohol. A skin circle of 2 cm in diameter and 2 mm in depth was removed from the area of dorsal neck. Excisions in the tissues underneath the wound area were avoided and the skin tension was kept constant during the experiment. The neck area was chosen for this test to prevent the rats from biting and stretching the wound area (Rouhollahi et al., 2015).

3.2.8.2 Topical application of the treatments

Rats in Negative control group were topically treated with 0.2 mL of the vehicle (2% CMC /20% glycerol v/v) once daily. Positive control group rats were treated once daily with 0.2mL of Intrasite gel topically. Animals of TIBH LD and TIBH HD groups were

treated once daily with 0.2 mL of the 10 or 20 mg/kg of TIBH in vehicle respectively. The wounds were daily monitored until the completion of the experiment.

3.2.8.3 Evaluation of wound closure percentage

The required period to form a scar at wound area without any remaining raw wound was termed the epithelization period. Based on the former similar studies, this period takes 10 to 20 days (Abood et al., 2015, Morton & Malone, 1972, Rouhollahi et al., 2015). In this study, wounds were photographed and wounds area were copied on transparent tracing paper respectively on day 0, 5, 10 and 15 of the experiment. The wound area was then measured using Image J (1.50i). The wound closure percentage (WC %) was calculated using the subsequent formula where WA (cm2) was the measured wound area (Suzuki-Banhesse et al., 2015)

(WC %) = [(WA at day 0 – WA at day X) / WA at day 0] × 100%

3.2.8.4 Histopathological evaluation of wounds

On day 15, after euthanasia of the animals with overdose of 150 mg/kg of ketamine (100mg/mL) and 15 mg/kg of Xylazil (100 mg/mL) intramuscularly the wound tissues with normal skin adjacent to wound site were excised. Some of the tissues were fixed in 10% of phosphate buffered formalin and processed for histological method and the rest were preserved for tissue homogenate. Fixed tissues were processed, paraffin embedded, sectioned and stained with haematoxylin & eosin and masson‟s trichrome (Appendix C & E). They were then examined under light microscope. Images analysis has been done by two experienced observers in an independent and blinded fashion for the control and the experimental groups (Amin et al., 2015).

3.2.8.5 Evaluation of Bax expression in wounds

The procedure was explained in sections 3.2.7.6.

3.2.8.6 Evaluation of Lipid peroxidation

Cayman‟s TBARS assay kit was used to detect the Malondialdehyde (MDA) (An end product of lipid peroxidation). A total of 100 µL supernatant of homogenized negative control, positive control and TIBH-treated group‟s tissues were separately mixed with 100 µL of SDS solution and 4 mL of the colour reagent then boiled (for 1 hour). After that the reaction was immediately ended by transferring the mixture into the ice bath (for 10 min). The mixture was centrifuged for 10 min at 1,600g at 4^{o}C. A total of 150 µl of the mixture was added to the 96 well plate in triplicate and the absorbance was read at 532 nm. MDA standard curve was plotted and the MDA value of each sample were calculated from standard curve.

3.2.8.7 Evaluation SOD activity

Activity of the superoxide dismutase (SOD) in the supernatant of all the scar tissue homogenates was measured using a Cayman‟s assay kit as stated by the kit‟s instructions. SOD samples plus with the SOD standard were set up in the assay plate. Briefly, 10 µL of assay standard and 10 µL of the supernatant of negative control, positive control and TIBH-treated group‟s tissue homogenate were separately mixed with 200 µL of diluted radical detector in the wells. Then 20 µl of diluted xanthine oxidase was added to the all wells. After 30 min incubation on the shaker, the absorbance was measured at 440–460 nm.

3.3 Statistical Analysis

All results were recorded as mean ± S.E.M. The statistical analysis of differences between negative control and experimental groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey"s post hoc comparisons. IBM SPSS software (SPSS ver.20) was used. In *in vitro* assays, each value represents mean of the three replicates. In acute toxicity assay, TIBH treated groups were compared with normal control group. In ulcer prevention study, obtained data from the TIBH pre-treated animals were compared with the obtained data from the ulcer control group animals. In wound healing study, obtained data from the TIBH treated groups were compared with the obtained data from negative control group.

CHAPTER 4: RESULTS

4.1 Antioxidant Activity

4.1.1 DPPH Scavenging Activity

DPPH radical scavenging result was shown in Figure 4.1. TIBH showed a remarkable DPPH radical scavenging activity, with IC_{50} values of 33.8 µg/ml, when compared to reference controls (ascorbic acid and quercetin) with IC_{50} values of 77.8 and 138 µg/ml, respectively. It means at any specific concentration, TIBH captured more DPPH formed free radicals and has a significantly higher antioxidant activity compared to ascorbic acid and quercetin. Each DPPH radical scavenging value represents mean of the three replicates. Coefficient p-values for the regression line of DPPH radical scavenging activity versus various concentration of ascorbic acid, quercetin and TIBH was significant.

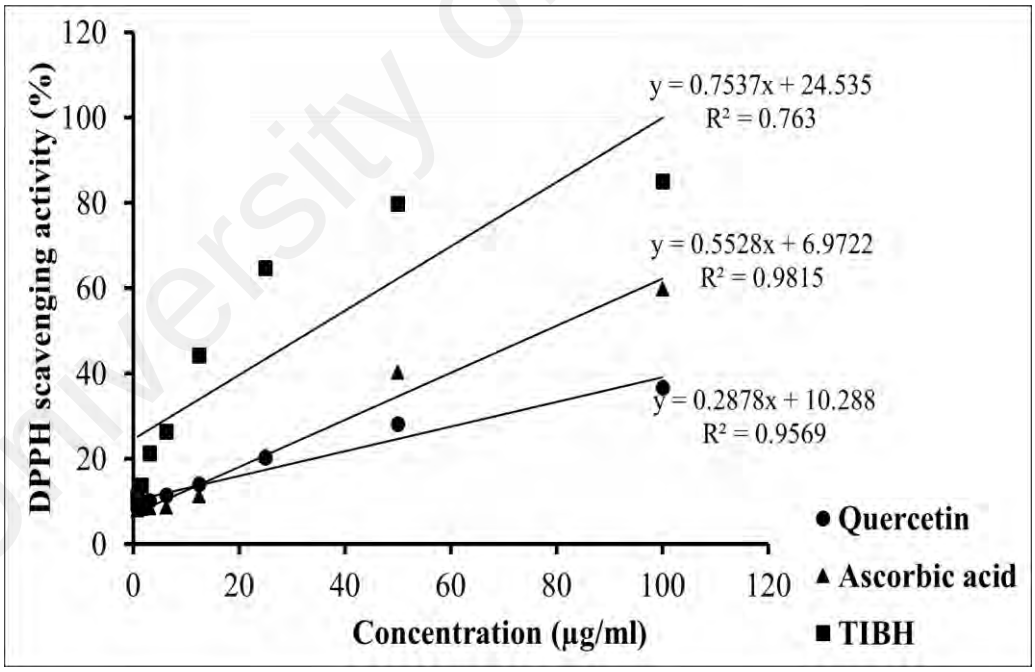

Figure 4.1: DPPH radical scavenging activity of various concentrations of TIBH, ascorbic acid and quercetin.

4.1.2 Frap Value

The reducing power estimated by the FRAP assay (Figure 4.2) displayed the TIBH offered more reducing power (p, 0.01) than the ascorbic acid and quercetin. The efficacy of the compound to transfer an electron to the TPTZ-Fe3+ is directly related to its antioxidant potential. According to the obtained results, the slope of the TIBH dose–dependent frap line was 20 times higher than the slope of ascorbic acid frap line and was almost same as the slope of quercetin frap line. So the compound sowed a very strong FRAP at the evaluated concentrations compared to positive controls. Coefficient p-values of FRAP regression line for ascorbic acid, quercetin and TIBH was significant. Each point represents the mean of the three readings.

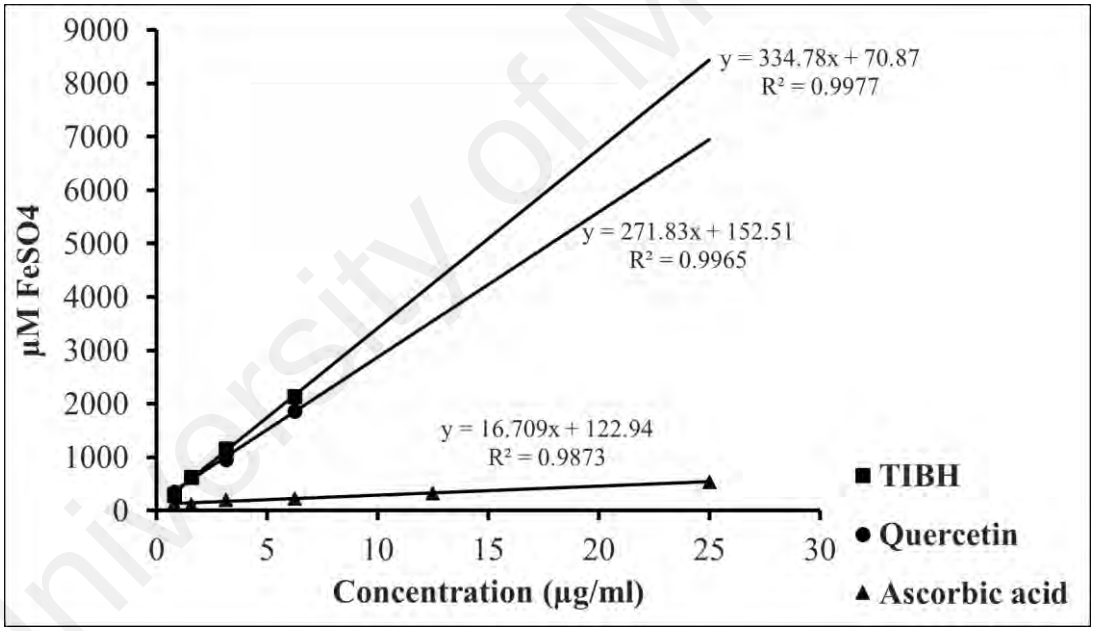

Figure 4.2: Dose–dependent plot of TIBH, ascorbic acid and quercetin in the ferric reducing/antioxidant power (FRAP) assay.

4.2 Cytotoxicity

DMSO cytotoxicity assay against human normal fibroblast cells showed that DMSO was not toxic to the cells at concentration of 1% and below (Figure 4.3). So DMSO in concertation less than 1% (V/V) was used to solve the TIBH. TIBH cytotoxicity assay result showed that TIBH in concentration less than 154 µM (50 µg/ml) was not toxic against human normal fibroblast and TIBH IC50value for cytotoxicity was equal to77.4 µM (25.18 µg/ml). Coefficient p-value of regression line of cell viability (%) versus various TIBH concentrations was significant (Figure 4.4).

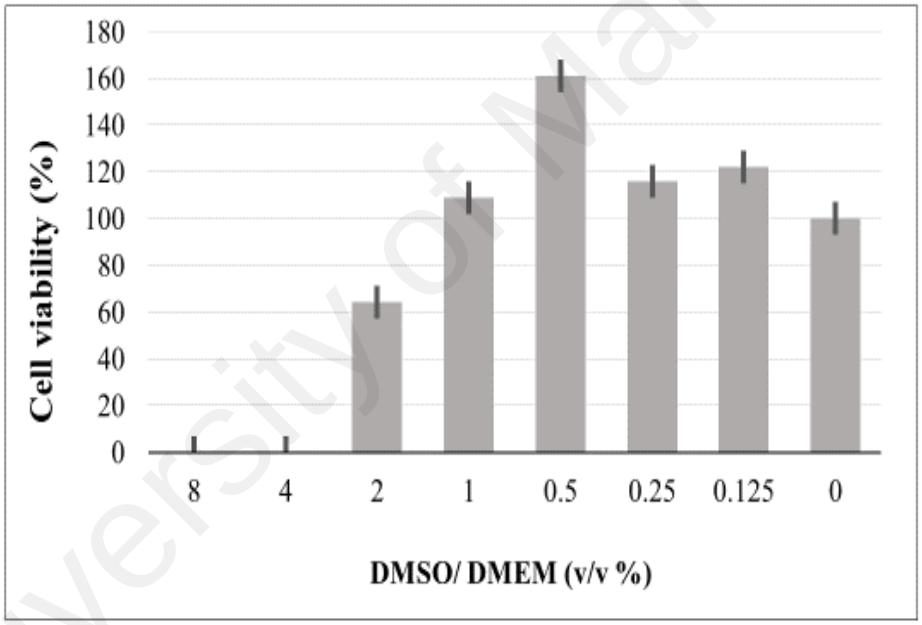

Figure 4.3: Cytotoxicity effect of the DMSO against human normal fibroblast cell line (BJ-5ta).

The result showed DMSO in concentration less than 1% in media was not toxic against human normal fibroblast. No cell growth was resulted at the 8% and 4% DMSO concentration. Each point represents the mean of the three readings.

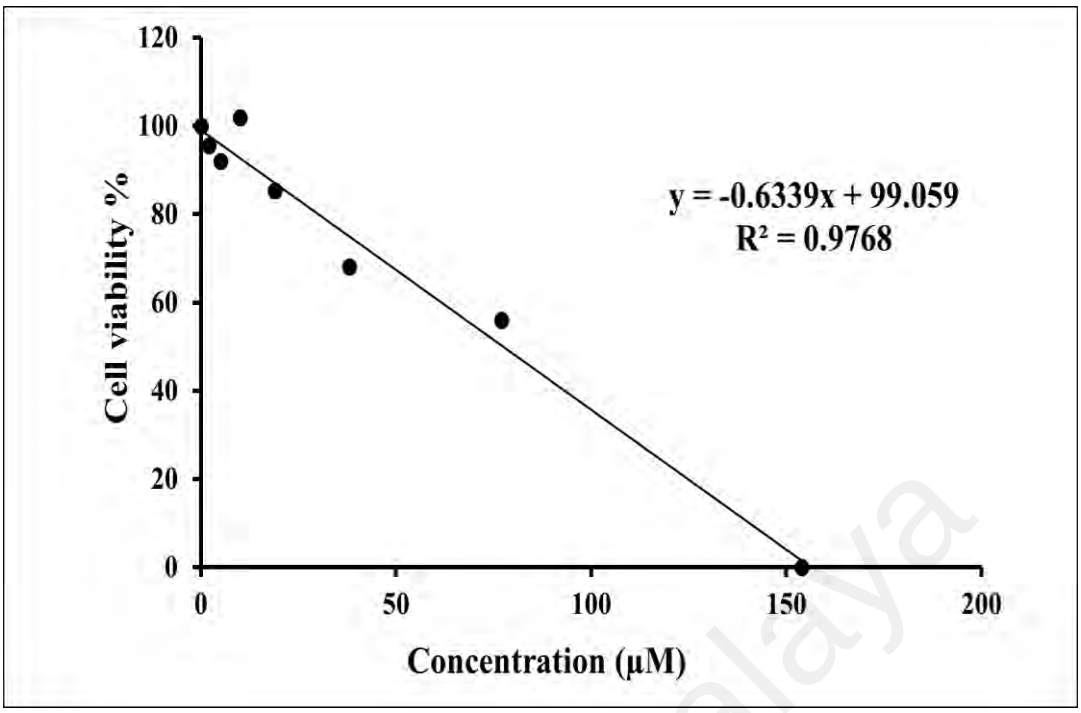

Figure 4.4: Cytotoxicity effect of the TIBH against human normal fibroblast cell line (BJ-5ta).

The result showed TIBH in concentration more than 154 µM (50 µg/ml) was toxic against human normal fibroblast and the TIBH IC50 value obtained from the graph equals to77.4 µM (25.18 µg/ml). Each point represents the mean of the three readings.

4.3 Antimicrobial Activity

All test tubes contained the inoculation of the bacteria showed the maximum McFarland value of 4 while the sterility control tubes had McFarland value of 0. The results indicated that there was no growth in the sterility control tubes (without bacterial inoculation) while the growth occurs in all bacterial suspension with various concentration of the TIBH. The obtained MIC for Chloramphenicol was 50µg/ml. TIBH MIC against two strains of gram positives Staphylococcus aureus and g negatives

Escherichia coli was recorded greater than the highest tested concentration of the TIBH (200 µg/ml).

4.4 Acute Toxicity

No significant abnormality, toxicity or death was observed among the experimental groups upon the oral gavage of 300 and 2000 mg/kg of TIBH in 0.1% CMC during 15 days of the experiment. Histological analysis of liver and kidney showed no hepatotoxicity or nephrotoxicity of the rats received 300 and 2000 mg/kg of the TIBH compared to the control group (Figure 4.5). Kidney (Table 4.1a) and liver (Table 4.1b) function hematological parameters analysis did not reveal any significant differences among groups.

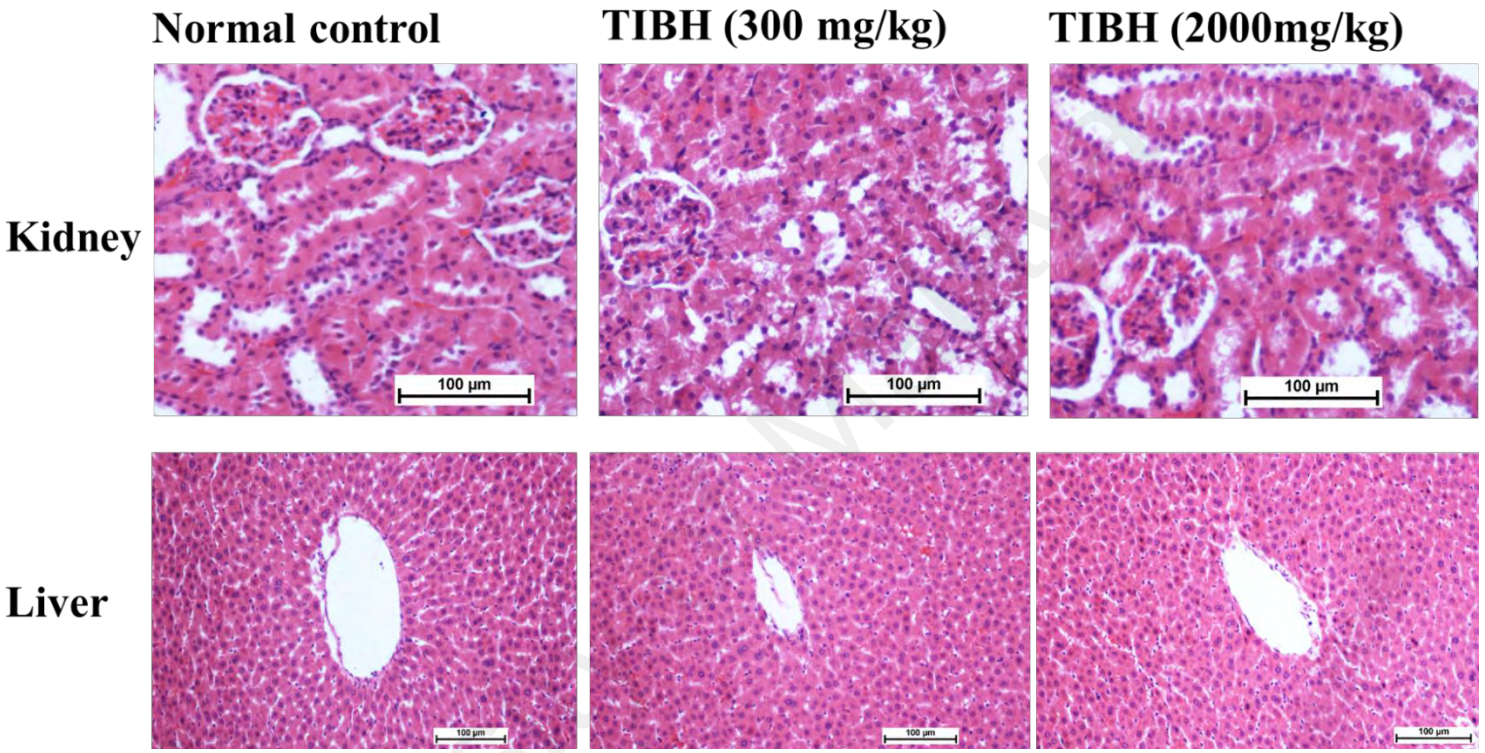

Figure 4.5: Histology of Kidneys and liver of rats in acute toxicity study (H&E staining, 20 x),(n=6).

Table 4.1 a: Effects of TIBH on kidney function parameters in acute toxicity test in rats

Animal groups	**Sodium (mM/L)**	**Potassium (mM/L)**	**Chloride (mM/L)**	**Carbon dioxide(mM/L)**	**Urea (mM/L)**	**Creatinine (µM/L)**
Normal control	141.32 ± 0.33	5.40 ± 0.61	101.45 ± 0.88	31.67 ± 0.88	8.1 ± 0.68	27.67 ± 1.43
TIBH (300 mg/kg)	142.56 ± 0.37	5.43 ± 0.26	102.98 ± 0.33	33.20 ± 0.51	7.41 ± 0.73	27.33 ± 0.67
TIBH (2000mg/kg)	143.09 ± 0.51	5.5 ± 0.65	103.75 ± 0.88	32.05 ± 0.65	9.3 ± 0.47	28.58 ± 1.0

Values stated in mean ± SEM. The results did not show any significant difference between the groups (n = 6). Significant considered as $p < 0.05$.

Table 4.1 b: Effects of TIBH on Liver function parameters in acute toxicity test in rats

Animal groups	Albumin (g/L)	Total bilirubin (µM/L)	Alkaline phosphatase (IU/L)	Alanine amino Transaminase (IU/L)	G-Glutaml. Transferase (IU/L)
Normal control	40.69 ± 0.88	< 2	232 ± 9.87	64.25 ± 7.8	-1.3 ± 0.6
TIBH (300 mg/kg)	43.35 ± 1.2	< 2	191 ± 12.73	65.67 ± 5.4	-2.1 ± 0.2
TIBH (2000mg/kg)	45.61 ± 1.45	< 2	205 ± 10.35	68.55 ± 8.68	-2.6 ± 0.5

Values stated in mean ± SEM. The results did not show any significant difference between the groups (n = 6). Significant considered as $p < 0.05$.

4.5 Ulcer Prevention Activity

4.5.1 Gastric Juice Acidity and Increased Gastric Mucus Production

Administration of the HCl/Ethanol increased the gastric acidity in ulcer control group comparing to the normal control group. Gastric acidity reduced significantly in rats pre-fed with TIBH compared with the rats of the ulcer control group and the resulted pH was comparable for the omeprazole pre-treated rats (Table 4.2). The gastric mucus content was significantly increased in the TIBH high dose and medium dose pretreated rats compared to the ulcer control rat (Table 4.2).

Table 4.2: Effects of the TIBH on gastric pH, mucus and ulcer area against HCl/ethanol-induced gastric ulcer

Animal Groups	**Pre-treatment (5ml/kg)**	**Gastric pH**	**Mucus Weight (g)**	**Ulcer area (mm^2)**	**Inhibition (%)**
Normal control	1% CMC	5.8 ± 0.2	0.89 ± 0.03	0	0
Ulcer control	1% CMC	3.2 ± 0.25	0.41 ± 0.01	246.4 ± 35	0
Positive control	Omeprazole in (20mg/kg)	7.2 ± 0.22*	0.7 ± 0.05*	31.6 ± 4*	87.2
TIBH LD	TIBH (50mg/kg)	6.4 ± 0.24	0.46 ± 0.02	111.2 ± 9*	54.9
TIBH MD	TIBH (100mg/kg)	6.8 ± 0.31*	0.68 ± 0.04*	42.0 ± 2*	83
TIBH HD	TIBH (200mg/kg)	7.2 ± 0.35*	0.77 ± 0.06*	24.4 ± 2*	90.1

„Table 4:3, continued" All values are expressed as mean ± SD (n = 6). * Shows the significant value was set at $p < 0.05$, compared with ulcer control group.

4.5.2 Gastric Ulcer Area

The macroscopic appearance of the stomach in TIBH pre-treated groups and control groups are shown in Figure 4.6. Ulcer area and the inhibition percentage of all groups are presented in Table 4.2. The rats pre-fed with the TIBH showed a significant reduction in ulcer area when compared to the ulcer control. Pretreatment of rats with the TIBH diminished the ulcer area generation which was comparable to the supportive effect of omeprazole.

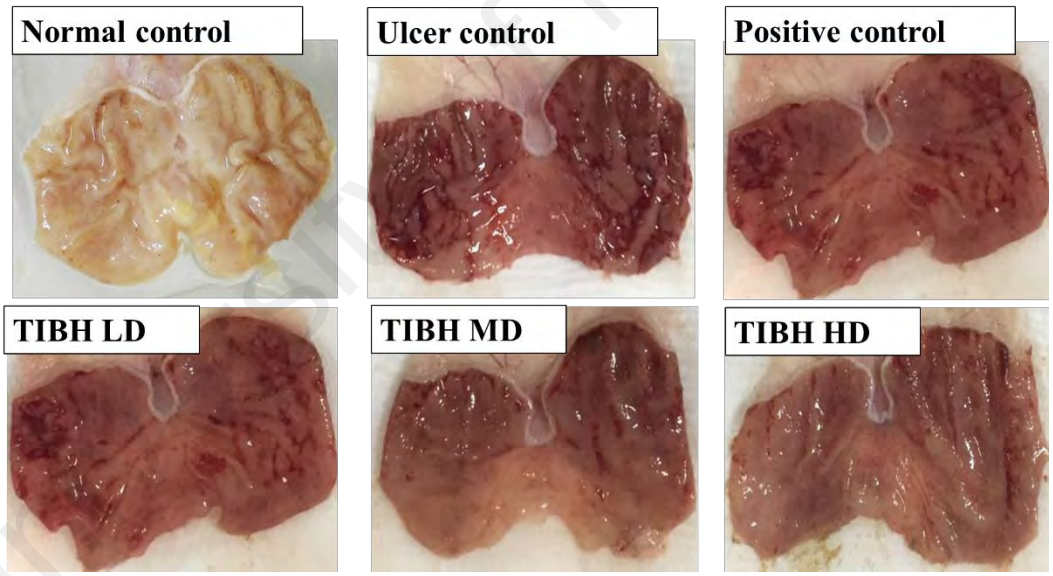

Figure 4.6: Effect of TIBH on macroscopic appearance of rat"s stomach against HCl/ethanol-induced ulcer.

Normal control group did not display any lesions in the gastric mucus membrane; In ulcer control group, strong ulceration was generated in the stomach; Positive control group (Omeprazole, 20 mg/kg) displayed minor gastric damages; low dose group pre-

treated with 50 mg/kg of the TIBH also displayed moderate gastric damages; Medium dose and High dose pretreated groups with respectively 100 and 200 mg/kg of the TIBH revealed decrease of the gastric damages in comparison with the ulcer control. Each group had 6 animals (n=6).

4.5.3 Histopathological Effects in Ulcer Prevention

4.5.3.1 H & E staining

Microscopic analysis of the H&E stained gastric tissues revealed that ulcer control group possessed substantial injuries of the gastric mucosa and in some areas gastric submucosa layer was associated with leucocytes infiltration and severe edema comparing to the TIBH pre-treated groups. TIBH pretreated groups displayed a gastric mucosal protection and a decline in edema and leucocytes infiltration of the submucosal layer (Figure 4.7).

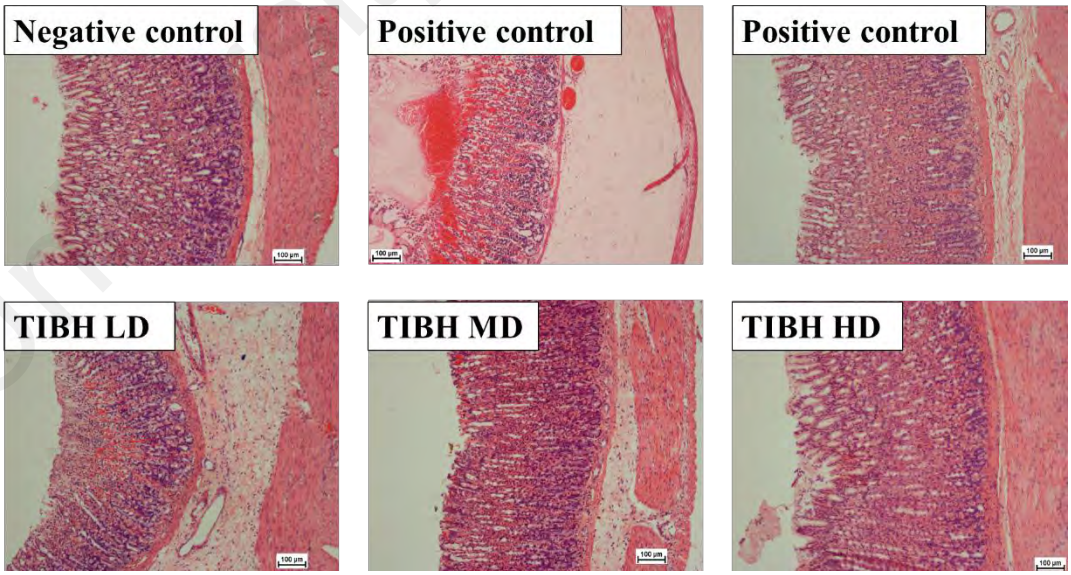

Figure 4.7: Histological analysis of gastric tissues using hematoxylin and eosin staining, 10 X magnification.

4.5.3.2 PAS staining

There was an increase in PAS staining of the gastric mucosa in rats pretreated with TIBH while compared with the rats of ulcer control group, which specified an increase in the glycoprotein content of the gastric mucosa (Figure 4.8), indicated that TIBH increases gastric mucus secretion.

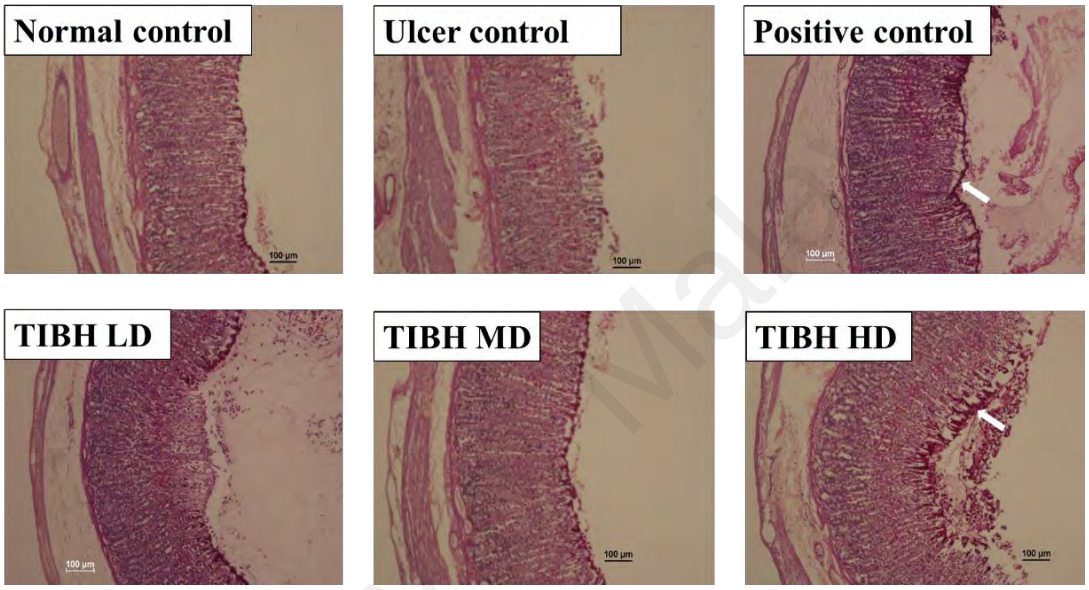

Figure 4.8: Histological analysis of gastric tissues using PAS staining, 10X.

High dose TIBH pre-treated group showed increment of magenta color (white arrow) compared to ulcer control groups which indicated the increase of the secreted mucus by gastric glands.

4.5.3.3 Immunostaining

Immunostaining results indicated that rats fed with TIBH had over-expression (brown color) of HSP70 protein compared to ulcer control group (Figure 4.9). Immunohistochemical staining of Bax protein demonstrated that rats pretreated with

TIBH had a decrease in the expression of Bax protein (Figure 4.10). Over-expression of HSP70 and Bax were indicated by brown color.

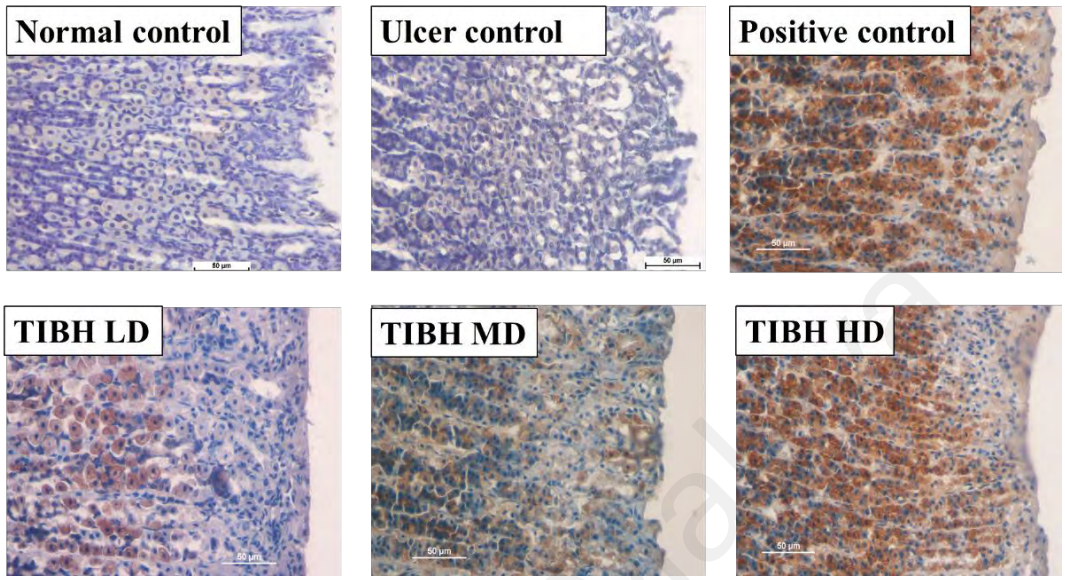

Figure 4.9: Immunohistochemical analysis of HSP70 protein in gastric tissues.

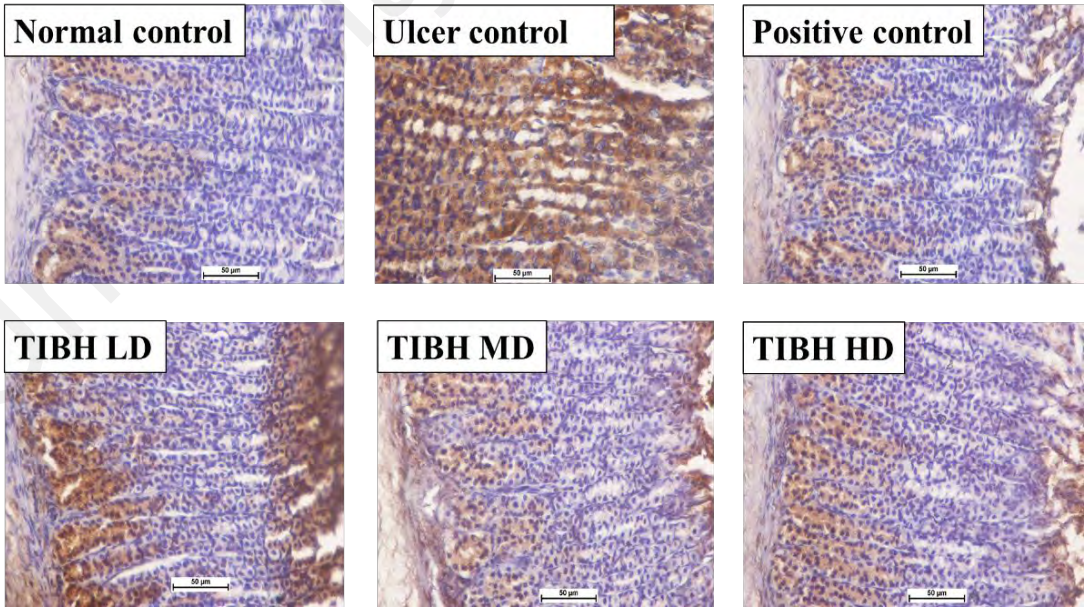

Figure 4.10: Immunohistochemical analysis of Bax protein in gastric tissues.

4.5.4 Lipid Peroxidation and SOD Activity

The MDA level in the gastric tissues homogenate of the TIBH pre-treated groups was significantly decreased compared to the ulcer control group (Table 4.3). SOD activity was significantly enhanced in the TIBH pre-treated groups in comparison to ulcer control (Table 4.3).

Table 4.3: Effect of TIBH on MDA and SOD in tissue homogenates

Animal Groups	**Pre-treatment (5ml/kg)**	**MDA (nmol/ml)**	**SOD (U/µl)**
Normal control	1% CMC	17.43 ± 0.41	352 ± 9.15
Ulcer control	1% CMC	43.59 ± 0.83	222 ± 6.7
Positive control	Omeprazole (20mg/kg)	32.85 ± 0.76*	321 ± 8.1*
TIBH LD	TIBH (50mg/kg)	40.20 ± 0.93	230 ± 7.25
TIBH MD	TIBH (100mg/kg)	30.80 ± 0.93*	280 ± 5.1*
TIBH HD	TIBH (200mg/kg)	29.26 ± 0.49*	306 ± 7.2*

All values are expressed as mean ± SD (n = 6). * Shows the significant value was set at $p < 0.05$, compared with ulcer control group.

4.6 Wound Healing Activity

4.6.1 Wound Closure Percentage

Grossly, the wounds dressed with TIBH 10 mg/kg revealed a remarkable wound repair and the rate of healing significantly accelerated compared to negative control group. Positive control group had the highest rate of healing among all groups. Wounds dressed with 10 mg/kg of TIBH achieved a wound-healing rate equivalent to the healing rate of positive control group. Rats treated with 20 mg/kg of TIBH had a faster wound-healing rate than rats in negative control group but a slower wound-healing rate than rats in intrasite gel and 20 mg/kg TIBH-treated group (Figure 4.11). Wound closure was measured to determine the percentage of wound healing in each rat (Table 4.4). Percentage of wound closure in the CMC/glycerol treated group (Negative control) was significantly lower when compared to TIBH low dose or Intrasite gel treated groups.

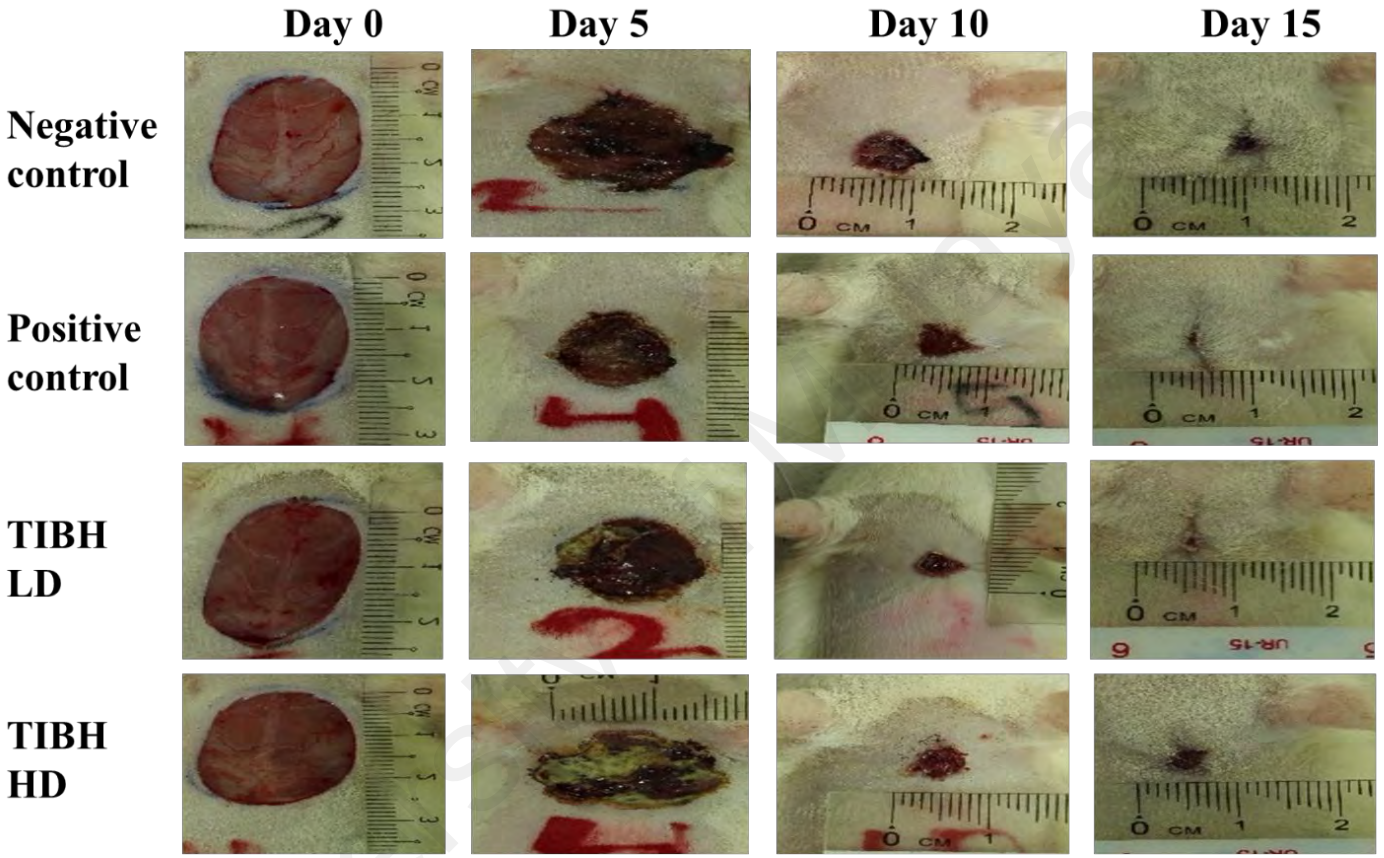

Figure 4.11: Effect of TIBH on the gross evaluation of wound healing on day 0, 5, 10 and 15. Negative control) 2% CMC / 20% glycerol v/v (vehicle); Positive control) Intrasite gel; TIBH LD) 10 mg/kg; TIBH HD) 20 mg/kg of TIBH.

Table 4.4: Effect of TIBH on wound closure percentage (%) in experimental rats

Groups	**Day0** **Wound area (cm2)**	**Day5** **Wound area (cm2)**	**Closure %**	**Day10** **Wound area (cm2)**	**Closure %**	**Day15** **Wound area (cm2)**	**Closure %**
Negative control	3.77 ± 0.03	2.24 ±0.07	40.64 ± 2.08	0.46 ± 0.05	87.89 ± 1.35	0.16 ± 0.02	95.88 ± 0.51
Positive control	3.74 ± 0.15	1.49 ± 0.06*	60 ± 1.48*	0.24 ± 0.02*	93.7 ± 0.44*	0.04 ± 0.03*	98.91 ± 0.94*
TIBH LD (10 mg/kg)	3.67 ± 0.04	1.57 ± 0.03*	57.15 ± 0.6*	0.28 ± 0.01*	92.27 ± 0.35*	0.02 ± 0.0*	99.54 ± 0.30*
TIBH HD (20 mg/kg)	3.83 ± 0.19	1.88 ± 0.09*	49.97 ± 4.58*	0.44 ± 0.03	88.25 ± 1.25	0.08 ± 0.04	97.44 ± 0.91

Mean values ± SEM and n=6 animals in each group were used. Significance was defined as $* P < 0.05$ compared to negative control group.

4.6.2 Histopathological Effects in Wound Healing

Wound sections were histologically examined using H & E and Masson‟s trichrome staining.

4.6.2.1 H & E staining

(a) ***2 x magnification***

Figure 4.12 shows histological analysis of wound tissues on day 15 after staining with hematoxylin and eosin, observed with 2X magnification.

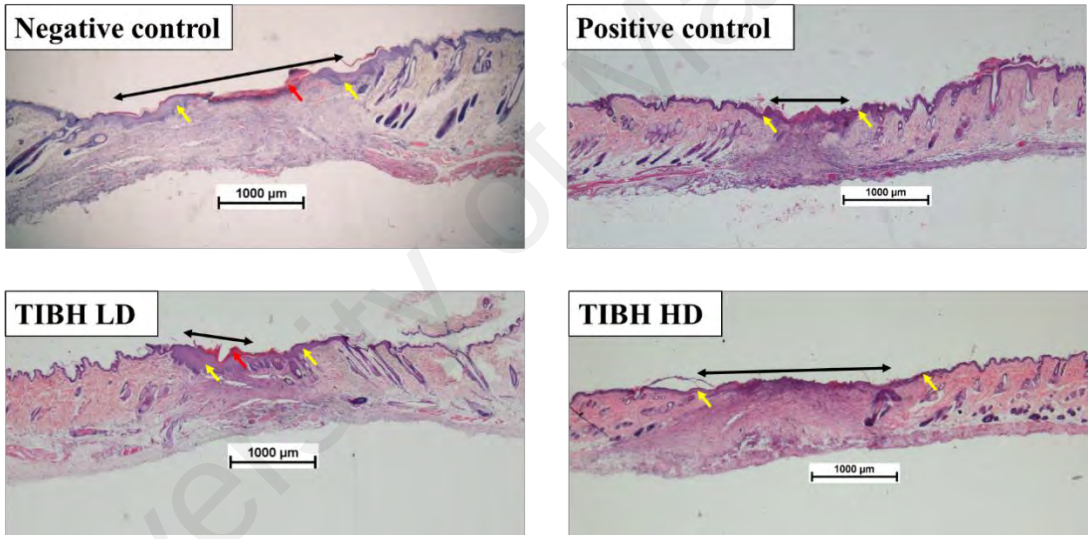

Figure 4.12: Histological analysis of wounds tissues on day 15 using hematoxylin and eosin staining (2X magnification).

Negative control (2% CMC / 20% glycerol v/v) showed a wide wound area (black arrow); Positive control group (Intrasite gel) showed smaller wound area (black arrow) compared to negative control; TIBH LD (10 mg/kg) likewise showed a small wound area (black arrow), TIBH HD (20 mg/kg) showed a moderate wound area compared to negative control. Red arrow = scab, yellow arrow = epidermis.

(b) ***100 x magnification)***

Figure 4.13 shows histological analysis of wound tissues on day 15 after staining with hematoxylin and eosin, observed with 100 X magnification.

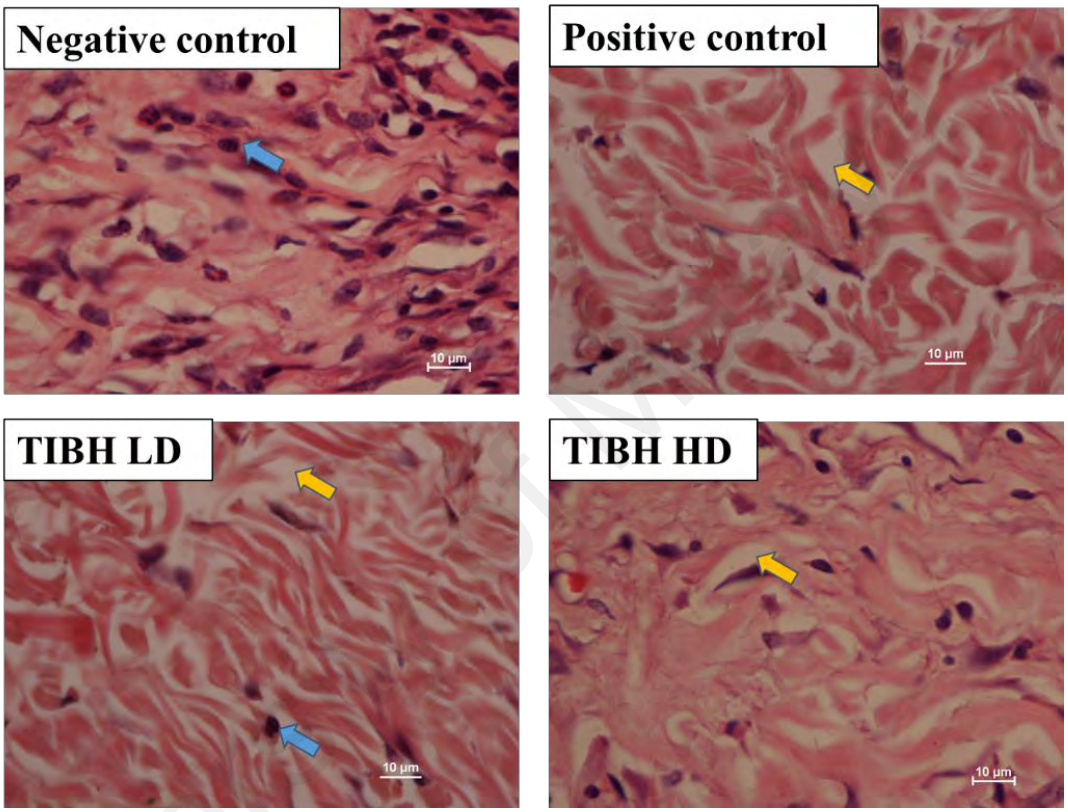

Figure 4.13: Histological analysis of wounds tissues on day 15 using hematoxylin and eosin staining (100 X magnification).

Negative control (2% CMC / 20% glycerol v/v) showed a wide wound area (black arrow); Positive control group (Intrasite gel) showed smaller wound area (black arrow) compared to negative control; TIBH LD (10 mg/kg) likewise showed a small wound area (black arrow), TIBH HD (20 mg/kg) showed a moderate wound area compared to negative control. Red arrow = scab, yellow arrow = epidermis.

4.6.2.2 Masson's trichrome staining

Figure 4.14 shows histological analysis of wounds tissues on day 15 using Masson‟s trichrome staining.

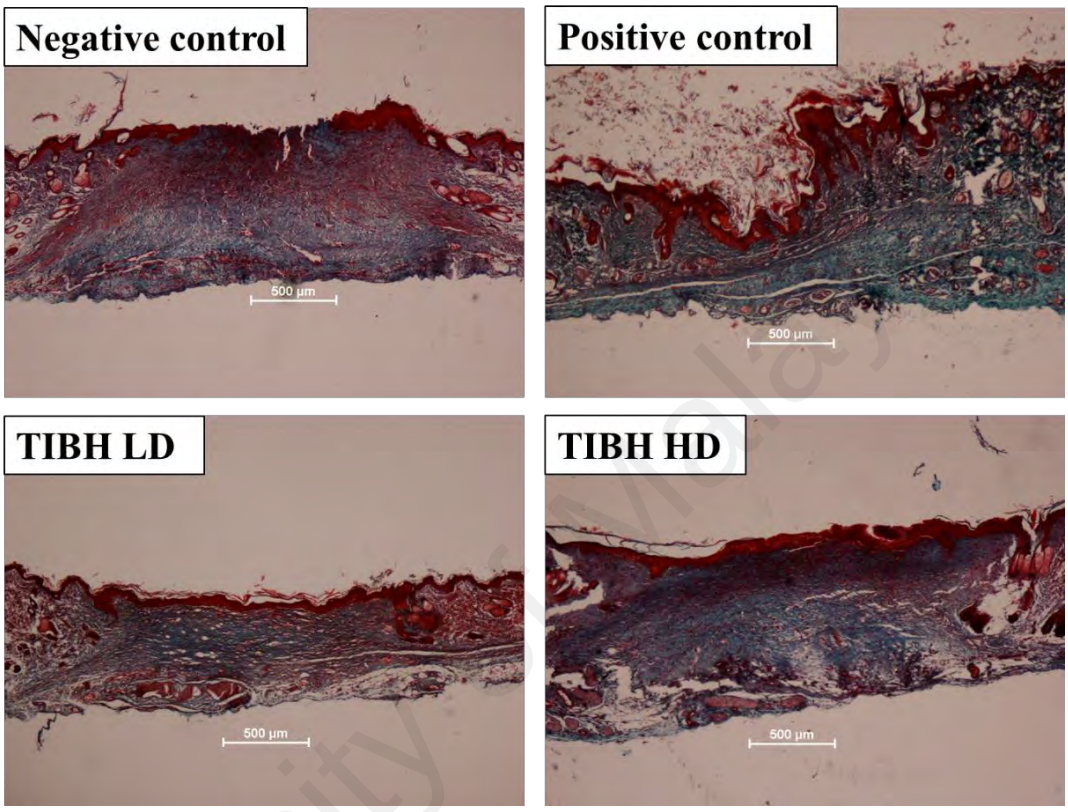

Figure 4.14: Histological analysis of wounds tissues on day 15 using Masson‟s trichrome staining.

Negative control (2% CMC / 20% glycerol v/v) showed many inflammatory cells, least number of fibroblasts and little amount of collagen disposition; Positive control group (Intrasite gel) showed remarkably more collagen fibres and fibroblasts with less inflammatory cells compared to negative control; TIBH LD group (10 mg/kg) showed noticeably less inflammatory cells and more fibroblast and collagen deposition; TIBH HD group (20 mg/kg) showed moderate number of inflammatory cells, a few number of fibroblasts and little amount of collagen disposition compared to negative control. Blue arrow = inflammatory cells, yellow arrow = collagen.

4.6.2.3 Immunostaining (Bax protein expression)

Immunohistochemistry staining of Bax protein on the other hand demonstrated that wounds treated with vehicle had an increase in the expression level of the Bax protein. Bax expression level decreased upon the TIBH administration of 10 mg/kg concentration and the results was comparable with intrasite gel treatment (Figure 4.15).

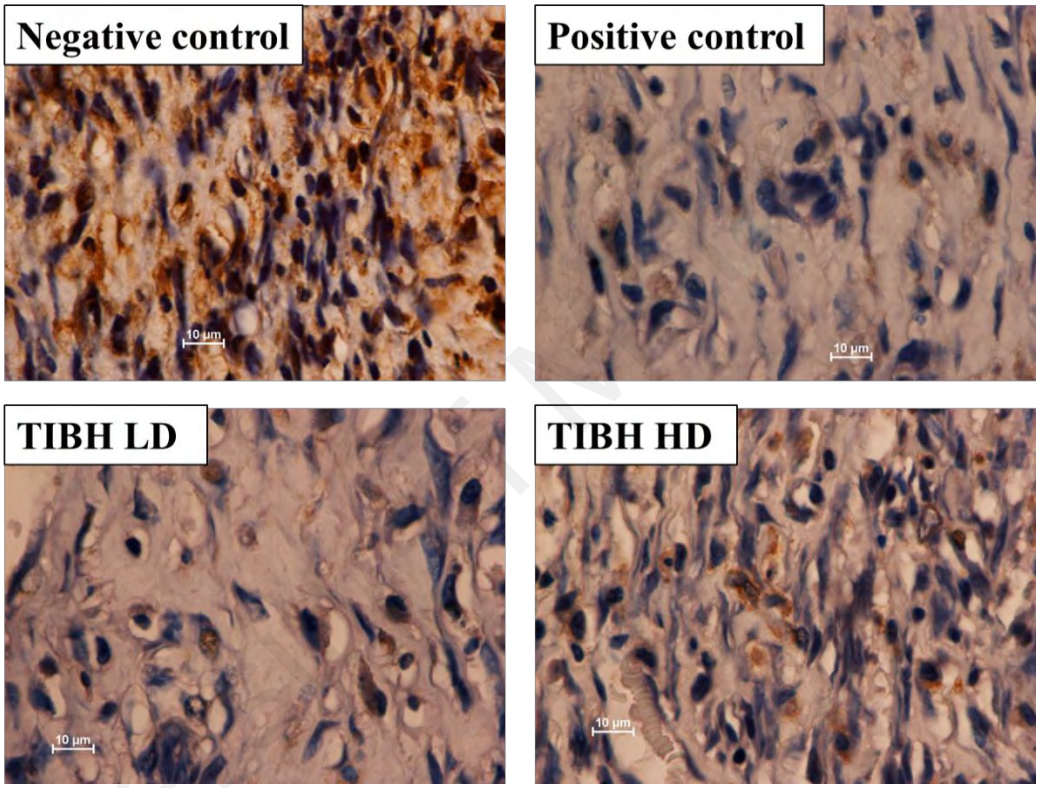

Figure 4.15: Immunohistochemical analysis of Bax protein in wounds tissues. Immuno-positivity of Bax protein was represented in brown color staining.

The results demonstrated that rat‟s wounds topically treated with TIBH LD (10mg/kg) showed a decrease in Bax protein expression level when compared to the vehicle treated wounds.

4.6.3 Lipid Peroxidation and SOD Activity

Topical treatment of wounds with TIBH low dose and intrasite gel significantly reduced MDA level when compared to the vehicle alone treatment (negative control group) (Figure 4.16). This result suggests that TIBH treatment at low doses remarkably decreased the lipid peroxidation in the rats wound area. Figure 4.17 shows the alterations of the SOD activity in the wound area of the treated rats. Results showed that topical treatment of rats wound with TIBH (10mg/kg) and intrasite gel significantly increased the activity of SOD in the wound tissues when compared to the wounds treated with vehicle.

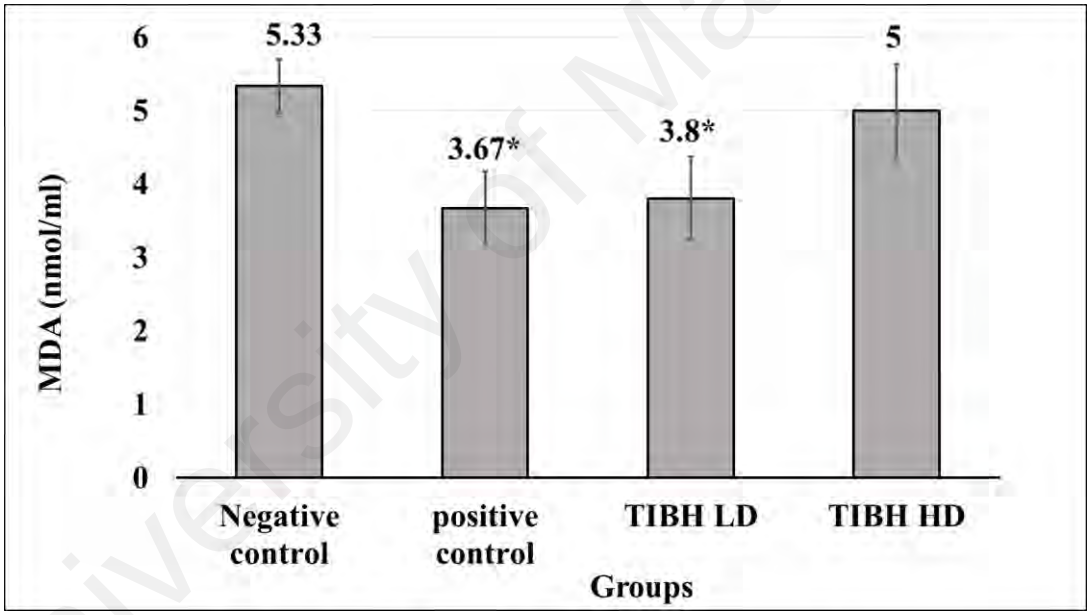

Figure 4.16: The MDA level in the wound tissues homogenate.

Negative control) 2% CMC / 20% glycerol v/v (vehicle); Positive control) Intrasite gel; TIBH LD) TIBH at dosage of 10 mg/kg of rats dissolved in vehicle; TIBH HD) TIBH at dosage of 20 mg/kg of rats dissolved in vehicle. Significance was defined as $*$ $P < 0.05$ compared to negative control group.

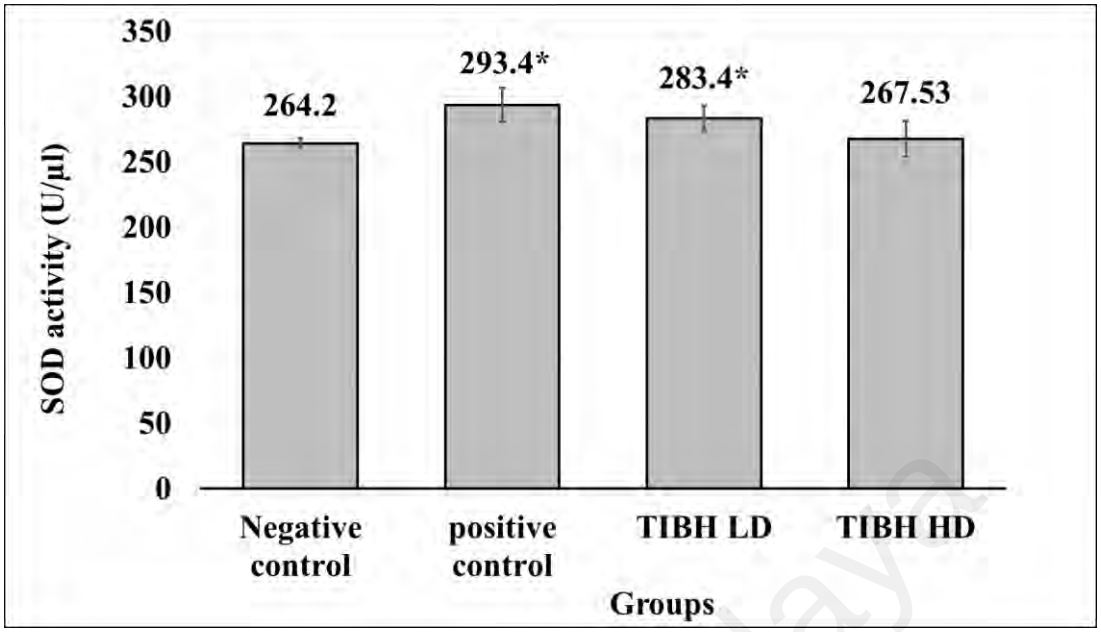

Figure 4.17: The SOD activity in the wound tissues homogenates.

Negative control) 2% CMC / 20% glycerol v/v (vehicle); Positive control) Intrasite gel; TIBH LD) TIBH at dosage of 10 mg/kg of rats dissolved in vehicle; TIBH HD) TIBH at dosage of 20 mg/kg of rats dissolved in vehicle. Significance was defined as $*$ $P < 0.05$ compared to negative control group. Each group had 6 animals (n=6).

CHAPTER 5: DISCUSSION

It has been confirmed that antioxidants play a substantial role in the maintenance of infected and non-infected wounds through decreasing oxidative stress generated damages (Geethalakshmi et al., 2013). Previous studies also showed that antioxidant compounds help to control wound oxidative stress and therefore accelerate wound healing (Fitzmaurice et al., 2011). Therefore In this study, the improvement of rat skin‟s wound healing due to the topical administration of TIBH is also suggested to be due to its strong antioxidant properties. Strong antioxidant activity of TIBH was proven by DPPH assay results. TIBH IC_{50} value (33.8 µg/ml) was nearly 2 times lower than ascorbic acid and 4 times lower than quercetin which are both known strong antioxidant chemicals. The results of this study that was in correlation with the formerly reported result (Khaledi et al., 2011). In this study ascorbic acid was used as a reference control antioxidant and its DPPH IC_{50} value was recorded to be equal to 77.8 µg/ml. Some of the previously reported ascorbic acid DPPH IC_{50} values were respectively 5.85 µg/ml (Kanimozhi & Prasad, 2009), 16 µg/ml (Mohamad et al., 2004), 50 µg/ml (Charrier et al., 2006), 62 µg/ml (Shirwaikar et al., 2006), 79.84 (Kaviarasan et al., 2008), 110.78 µg/ml (Rice-Evans & Miller, 1996) and 172.77 µg/ml (Khaledi et al., 2011). The obtained DPPH IC_{50} value for ascorbic acid in this study was found to be similar to the reported IC_{50} values by Charrier et al. (2006), Shirwaikar et al. (2006) and Kaviarasan et al. (2008) and was reported to be higher than quercetin.

DPPH assay is impressively affected by solvent impurities and changes of the pH and in general it is less robust than FRAP assay. Therefore many studies has used the FRAP assay for the general analysis of antioxidant activity of various materials and chemicals (Benzie & Strain, 1996; Gliszczyńska-Świgło, 2006; Henderson et al., 2015; Katalinić et al., 2004). Nonetheless, in this study, strong antioxidant activity of TIBH

was also confirmed by the result of the FRAP assay. TIBH various concentrations possessed remarkably higher FRAP values in comparison with reference controls.

Wound healing is a complex process of cellular and biochemical interactions involving various cells such as keratinocytes, fibroblasts and endothelial cells (Krishnamoorthy et al., 2012). The most important cells in this process are fibroblasts which are key cells for angiogenesis, epithelialization and collagen formation initiation. In the progressive trauma healing, many of fibroblasts mature into myofibroblasts that promote wound closure. (Bainbridge, 2013). This study showed TIBH in concentration less than 50 µg/ml was not toxic against human normal fibroblast and this concentration is likewise lower than the TIBH IC_{50} value for DPPH radical scavenging activity (33.8 µg/ml).

It was previously reported by Khaledi et al. (2011) that TIBH IC_{50} value for cytotoxicity against Colon cancer and Brest cancer cell lines were respectively 21.0 ±1.5 µM and 27.9±3.1 µM. However in our study TIBH IC_{50} value for cytotoxicity against human normal fibroblast cells was shown to be 77.4 µM which is far greater than the IC_{50} values for the mentioned cancer cell lines. This result can suggest that TIBH anticancer property might be promising that can be investigated by future studies.

DMSO is used to solubilize poorly soluble drugs (Da Violante et al., 2002) and here we used it as a solvent of TIBH in TIBH cytotoxicity assay. It was previously reported that DMSO at concentration of up to 10% did not produce any significant alteration and cytotoxicity in Caco2/TC7 cells while a later report showed that DMSO concentrations between 0.5% and 6% and above resulted in a dose-dependent damaging effects on Cochlear Organotypic cultured cells. The result of our study showed that DMSO in concentration less than 1% in media was not toxic against human normal fibroblast which was comparable with result obtained by Qi et al. (2008). Therefore, in this study

to minimize the interference effect of DMSO with TIBH in cytotoxicity assay, DMSO concentration was kept below 1%.

The results of the toxicity test of this study indicated that the TIBH was not toxic upon the oral administration of 2000mg/kg. Similar results had been reported by several researchers using different compounds (Salga et al., 2011; Gwaram et al., 2012; Ketuly et al., 2013; Halabi et al., 2014).

Ulcer model of this study was generated using the HCl/Ethanol. The effects of ethanol on the gastric mucus membrane are damaging and dose-dependent with higher concentrations being more harmful. The injury occurs 30 minutes after the intake and is maximized at about 60 minutes later (Stermer, 2002). Acidification of ethanol enhanced the severity of the development of the gastric lesions compared with equivalent concentrations without HCI. HCl/Ethanol-induced peptic ulcer has been frequently used as a simulated model of peptic ulcer for the evaluation of the gastro-protective and ulcer treatment potential of the various chemicals (Asai et al., 2011; AlRashdi et al., 2012; Salama et al., 2016). The harmful effect of the HCl on gastric mucosa has been known since years ago (Mahl, 1950). Ethanol on the other hand rapidly penetrates to submucosa increases the generation of the reactive oxygen species (ROS) and decreases the mucus membrane secretion which together cause the damage of the gastric mucosal cells (Mustafa et al., 2009; Gwaram et al., 2012). Ethanol also inhibits cyclooxygenase and suppresses the secretion of prostaglandins from the non-parietal gastric epithelial cells (Tandon et al., 2004; Rouhollahi et al., 2014; Warzecha et al., 2014). Our study showed that administration of the TIBH significantly protects the gastric epithelium and suppresses the deleterious effects of the ethanol on the rat stomach gastric mucosa. This finding is consistent with previous studies (Al Batran et al., 2013; Hajrezaie et al., 2015; Salama et al., 2016). We found that TIBH pre-treatment decreased the HCl/Ethanol

produced ulcer area. The deduction of the damaged area caused by TIBH was comparable with healing effect of Omeprazole. Results derived from the macroscopic evaluation of the stomachs supported the protective effect of the TIBH against peptic ulcer and they were in agreement with the results from the previous studies (Ibrahim et al., 2012; Moghadamtousi et al., 2014; Salama et al., 2016).

Our results also presented that the oral administration of the TIBH before HCl/Ethanol administration significantly decreased the gastric acidity of the pre-treated groups compared to the ulcer control group. The capability to decrease the gastric acidity is considered to be a support for the treatment of the gastric ulceration and it has improved the management of peptic ulcer disease (Proctor & Deans, 2014). Omeprazole as a standard drug for peptic ulcer has shown healing effects against peptic ulcer due to its ability to minimize the degree of gastric acidity through the inhibition of the proton pumps in the parietal cells of the stomach (Brijnner et al., 1995; Golbabapour et al., 2013). TIBH high dose pretreatment had a comparable effect with the Omeprazole (positive control) pretreatment (Bancroft & Gamble, 2008; Liju et al., 2015; Nazarbahjat et al., 2016; Ibrahim et al., 2016).

Mucus is a protective gel layer attached to the mucosal surface. When the physiological conditions are normal, the mucus and bicarbonate defense mechanism is adequate to protect the gastric mucosa in contrast to acid and pepsin secretion. However, a number of agents such as ethanol and HCl can reduce the gastric mucus content and damage the gastric mucosa (Allen & Flemström, 2005; Hajrezaie et al., 2015). In our study, the mucus content of the stomach increased upon in the TIBH medium and high dose pre-treated groups compared to ulcer control group. PAS staining was also used to localize and measure the degree of mucus secretion as a histopathologic method. The Glycol functional groups presented in the mucus were

oxidized by periodic acid into dialdehydes which on reaction with Schiff's reagent gave an insoluble purple magenta reagent (Bancroft & Gamble, 2008; Johansson et al., 2013). Increase of the magenta color of stomach tissue in the high dose and medium dose TIBH pretreated groups compared to the control group indicated that the mucus secretion increased due to TIBH pretreatment. The PAS staining histology and the mucus content evaluation were in agree with each other and both confirmed the promoting effect of the TIBH on mucus formation as a barrier against HCl/Ethanol triggered mucosal necrotic damages. Former investigators showed similar results for mucus production promoting effect of other chemicals (Nordin et al., 2014; Hajrezaie et al., 2015; Ibrahim et al., 2016; Nazarbahjat et al., 2016).

Oxidative stress contributes to the formation of numerous syndromes such as peptic ulcers and gastric carcinoma (Tandon et al., 2004). Ethanol metabolism in the stomach releases hydroxyl and superoxide radicals. Former investigations have confirmed that lipid peroxidation is involved in the occurrence of acute gastric damages triggered by ethanol (Salga et al., 2012; Rouhollahi et al., 2014; Hajrezaie et al., 2015). A previous *in vitro* study showed that the TIBH has an inhibitory effect on lipid peroxidation (Khaledi et al., 2011). Our results furthermore indicated that TIBH exhibits protective activity against ethanol-induced oxidative stress. The results also specified that the gastro-protective effects of the TIBH on the HCl/Ethanol induced ulcer model in the rat is correlated with its ability to decrease the lipid peroxidation. Studies showed that the ethanol exert a destructive effect on the rat gastric mucosa by the generation of ROS resulting in the lipid peroxidation and decrease of the SOD activity (Kwiecien et al., 2002; Nazarbahjat et al., 2016). Excessively produced superoxide damages the surrounding tissues. Sufficient amount of SOD in the cells and tissues keeps the superoxide anions at a very low concentration (Bannister et al., 1987; Kurahashi & Fujii, 2015).A study demonstrated that the administration of the SOD to the rats could

protect their gastric mucosa against the induced injury by ethanol (Terano et al., 1989). Other studies showed that the SOD activity of the gastric tissue increased due to the exposure to various chemicals (Dhiyaaldeen et al., 2014; Hajrezaie et al., 2015; Nazarbahjat et al., 2016). In this study, SOD activity was significantly decreased due to excess production of superoxide anion by HCl/Ethanol administration. However, TIBH medium dose and high dose pretreatment could reverse the detrimental effects of the HCl/Ethanol on the gastric mucosa. Our study determined that the TIBH pretreatment has gastro-protective potential against the ROS induced gastric damages which is resulted from the HCl/Ethanol administration.

HCl/Ethanol administration resulted in the increase of the haemorrhagic ulcer, gastric submucosal oedema and infiltrated leucocytes (Nordin et al., 2014; Salama et al., 2016). Hemorrhagic ulcer occurs due to the mucosal vasodilation as a result of endothelin-1 release into the blood under the effect of the ethanol. Furthermore, ethanol produces apoptosis that induces the cell death (Gulia & Choudhary2011). It also destroys the blood vessel resulting in the increased vascular permeability, the edema formation and the epithelial cell loss. Hemorrhagic ulcer, epithelial cell loss, submucosal edema and infiltrated leucocytes were observed in the histopathological evaluation of the stomachs. Our results revealed that all of the symptoms were efficiently suppressed by TIBH medium and high dose pre-treatment as it was shown by former investigations (Gwaram et al., 2012; Hajrezaie et al., 2012; Ketuly et al., 2013; Halabi et al., 2014).

HSP70 protein is richly formed in the occurrence various forms of stress like heat, toxic substances, infection and proliferation (Shichijo et al., 2003). Bax stimulates apoptosis, while BCL-2 prevents it. Apoptosis happens to trigger due to the imbalance of the expression of BCL-2 family anti-apoptotic proteins and apoptotic Bax proteins (Emily et al., 2001; Konturek et al., 1999). Ethanol creates reactive oxygen species

(ROS) which down regulates the HSP70 and up-regulates the Bax. Bax and HSP70 proteins are accountable to support intercellular homeostatic mechanism. They inhibited physiologic damages by conserving the structure of other normal proteins and by repairing or eliminating impaired proteins. Up-regulation of HSP70 proteins and down-regulation of Bax proteins resulted in this research study suggested these regulations as gastro-protective mechanism of the TIBH against ethanol-induced hemorrhagic of gastric mucosal membranes in rat. The obtained results of the present investigation confirmed the previous results on the protection of the mucosal damages through the regulations of the Bax and HSP70 proteins (AlRashdi et al., 2012; Salama et al., 2016) and showed that TIBH has a potential role in prevention of gastric ulcer by regulating these proteins.

Macroscopic evaluation of wounds determined that topical application of TIBH (10mg/kg) on wound areas significantly improved the rate of the wound closure. This result was similar to the wound healing effect of some previously studied synthetic compounds and plant extras (Al-Bayaty & Abdulla, 2012; Abood et al., 2015; Amin et al., 2015).

Formation of a new epithelial layer is one of the most central procedures of the wound healing. Re-epithelialization happens when keratinocytes start to divide and occupy the wound surface (Evans et al., 2013). Histological analysis showed that TIBH treatment decreased the wound width and increased the reepithelialisation. Increment of angiogenesis in granulation tissues of the wound site enriches blood circulation which increased the transportation of the required oxygen and nutrients and enhances the proliferation of the various involved cells in the wound healing and helps in re-epithelialization. On the other hand collagen as a major component of mammal proteins in addition to the most abundant factor of granulation tissues enhances the tissue

regeneration. Collagen plays a substantial role in all phases of wound healing and it acts as a structural scaffold in tissues beside its recently discovered substantial functional roles (Brett, 2008). Histological analysis of TIBH revealed that the wound healing potential of TIBH might be due to its ability to increase the angiogenesis, increase the number of the fibroblasts and collagen deposition and to decrease the number of the inflammatory cells. Similarly other studies has reported that skin wound healing improvement increases with the increment of angiogenesis, collagen deposition and the decrement of the number of the inflammatory cells (Mahmood et al., 2010; Cheng et al., 2013; Hajiaghaalipour et al., 2013; Dhiyaaldeen et al., 2014)

Apoptosis occurs due to the imbalance of the expression of BCL-2 family anti-apoptotic proteins and apoptotic Bax proteins (Konturek et al., 1999, Emily et al., 2001). Bax expression increases markedly with the increment of apoptosis and shows a good association with the apoptosis in the process of wound healing (Cui et al., 2003). Dysregulation in apoptosis can result in wound healing complications, like development of hypertrophic scar and keloid (Moulin et al., 2004). Down-regulation of Bax proteins in TIBH-treated group compare to the vehicle-treated group in this study suggested this regulation as the possible protective mechanism for wound healing in rat and confirmed the previously reported studies (Moghadamtousi et al., 2015, Rouhollahi et al., 2015).

Antioxidants play a primary role in process of wound healing (Kurahashi and Fujii, 2015). Superoxide anions are the major ROS formed from molecular oxygen and they are rapidly detoxified by the action of SOD. In this study, local treatment of the wound with TIBH low dose significantly increased the SOD activity. Previous studies showed that SOD is beneficial in enhancing of wound healing. SOD mRNA was shown to be overexpressed in wound area as verified by an RNA protection assay and *in situ* hybridization (Steiling et al., 1999; Kurahashi and Fujii, 2015). It has been also reported

that a single regimen of a cutaneous gene therapy for SOD decreases the delay of healing of diabetic rats wounds (Luo et al., 2004). In our study, increase of the SOD activity might be one of the TIBH adopted strategy to enhance the wound healing through the decrease of the oxidative stress. Similarly, various former studied compounds and plant extracts were reported to accelerate the wound healing through their ability to increase the SOD activity (Abood et al., 2015; Rouhollahi et al., 2015).

Generation of lipid peroxidation due to ROS overproduction can delays wound healing (Altavilla et al., 2001). As a result, decrease of the lipid peroxidation would be a suggested mechanism in the improvement of wound healing. A previous *in vitro* study showed that the TIBH has an inhibitory effect on lipid peroxidation (Khaledi et al., 2011). In this experiment, wound tissue homogenate from rats treated with TIBH showed significant decrease in MDA level. Consequently, our results indicated that TIBH exhibits wound healing potential by decrease of the lipid peroxidation. Previous investigations also showed that enhancement of the wound healing by decreasing of the lipid peroxidation is a suggested path of action by some other chemicals (Altavilla et al., 2001; Abood et al., 2015; Rouhollahi et al., 2015).

DPPH and FRAP assays results showed that TIBH promotes free radical-scavenging activity and antioxidant activity. This is while measurement of the wound tissue homogenate enzymatic antioxidant activity and the MDA level showed that TIBH also increases the enzymatic antioxidant activity and decreases the lipid peroxidation. So it can be said that TIBH wound healing effect resulted in this study was due to TIBH both enzymatic and non-enzymatic antioxidants properties (Aboutwerat et al., 2003).

CHAPTER 6: CONCLUSION

In conclusion it can be said that TIBH treatment improved the wound healing by increasing of the fibroblast accumulation, collagen deposition, and endogenous antioxidant enzymes activity (SOD) and by the decreasing the number of inflammatory cells, lipid peroxidation (MDA) and Bax expression. TIBH showed a remarkable antioxidant activity as evaluated by DPPH and FRAP assays. Cytotoxicity assay revealed that TIBH concentration more than 50 µg/ml was toxic against human normal fibroblast with an IC_{50} value of 25.18 µg/ml. lastly antimicrobial assay showed that TIBH MIC against two strains of *Staphylococcus aureus* and *Escherichia coli* was greater than the highest tested concentration of the compound (200 µg/ml). TIBH is a safe compound in rats upon 2000 mg/kg oral administration. TIBH had a dose dependent ulcer prevention potential against HCl/Ethanol-triggered gastric ulcer which could be due to the increase of the mucosal secretion, decrease of the gastric acidity, up-regulation of HSP70 protein, down-regulation of Bax protein, decrease of the lipid peroxidation and the increase of the superoxide dismutase activity in gastric homogenate. Therefore, TIBH would be useful as gastric ulcer preventive compound, but future studies are needed to translate into use in human and clinical practice.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Tayeby, F., Salman, A. A., Kamran, S., Lay, K. S., Mohan, G. (2017). Ulcer prevention effect of 3,4,5-tihydroxy-n0-[(2-methyl-1h-indol-3yl)methylidene]benzohydrazide in hcl/ethanol-induced gastric mucosal damage in rats. *International Journal of Medical Sciences,* In Press. doi:10.7150/ijms.20984