WOUND HEALING AND GASTROPROTECTIVE EFFECTS OF *CLAUSENA EXCAVATA* LEAVES EXTRACT BY MODULATING INFLAMMATION AND OXIDATIVE STRESS

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ABSTRACT

Clausena excavata Brum. f. is an important traditional medicinal plant commonly used in Malaysia and South-East Asian countries for the treatment of many common ailments. However, the effect of *C. excavata* on wounds is not known. In this study, the wound healing and gastroprotective effects of *C. excavata* leaves extract were determined. Successive fractionation of leaves using petroleum ether (PT) followed by chloroform (CH), ethyl acetate (EA), and methanol (MOH) showed that MOH fraction possessed the highest total phenolic content of 522.0 ± 11.6 mg GAE/g plant extract. The ferric-reducing antioxidant power (FRAP) value of *C. excavata* MOH fraction was 4649 ± 11.3 µmol Fe^{2+}/mg, while the radical scavenging activity using 2, 2-diphenyl-1-picryl dihydrazyl (DPPH) was 86.89 ± 1.4 % with IC_{50} at 23 µg/mL. The MOH fraction was innocuous against the human keratinocyte (HaCaT), macrophage (J77A.1), and Vero cell lines when assessed by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay. Further, the acute toxicity study on rats indicates treatment with MOH fraction even at a high dose of 5000 mg/kg body weight did not cause mortality. Further, MOH did not affect the kidney and haematological parameters or relative weights of the liver or kidneys at a high dose of 5000 mg/kg body weight. The *in vitro* scratch test demonstrated that MOH fraction at 100 µg/mL significantly stimulated proliferation and migration of fibroblast cells (p<0.05) at 24 and 48 hours after treatment shown by the faster closure of wound area compared to the untreated cells. Topical treatment of wound-induced Sprague-Dawley rats with 50, 100 and 200 mg/mL MOH fraction daily for 15 days was found to be effective in stimulating wound healing by decreasing wound size, improving wound contraction, enhancing tissue regeneration and granulation tissue, which were more significant (P<0.05) compared to the untreated control rats. The wound healing effect of MOH fraction is attributed to the significant (P<0.05) increase in transforming growth factor-beta (TGF-β1), vascular endothelial growth factor (VEGF), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and interleukine 10 (IL-10), while a significant (P<0.05) decreased in cyclooxygenase-2 (COX-2), tumor necrosis factor alpha (TNF-α), interleukine 6 (IL-6) and lipid peroxidation (LPO). Histological evaluations using the haematoxylin and eosin and Masson trichome stain methods showed that healed skin after treatment with MOH fraction had less scarring and greater fibroblast proliferation, collagen deposition, and angiogenesis compared to the untreated control. Besides, MOH fraction provoked apoptosis by the down-regulation of BAX gene and up-regulation of Bcl-2 gene. To determine the gastroprotective effect of MOH fraction, oral ethanol-induced gastric ulcer and the pylorus ligature rat model were used. The acid volume and pH of the gastric juice were determined in the pylorus ligature model. MOH fraction at dose 200 and 400mg/mL also showed significant (P<0.05) anti secretory effect of gastric juice in the stomach. Pre-treatment with MOH fraction significantly (P<0.05) increased SOD, GPx and CAT and reduced LPO activities. Increased expression of heat shock protein70 (HSP70) and transforming growth factor beta (TGF- β1) proteins occurred while decreased expression of BAX protein occurred in the stomach tissues of MOH fraction-treated rats. MOH fraction of *C. excavata* leaves extract decreased ulcer area and leucocyte infiltration and edema of the gastric submucosal layer, decreased volume, increased pH, and decreased total acidity of the gastric juice, showing that it is gastroprotective . MOH was characterized by LCMS/MS and shown to contain, myricetin glucoside, quercetin-rhamnose-hexose, kaempferol, furocoumarin, and 8-geranyloxy psoralen. In conclusion, MOH fraction facilitates wound healing and exhibits protective effects against gastric ulcers by stimulating activity of tissue antioxidant enzymes, thus preventing free radical-mediated tissue injury and modulating inflammatory mediators, and regulating apoptosis.

ABSTRAK

Clausena excavata Brum. F adalah tumbuhan ubatan tradisional penting dan lazim digunakan di Malaysia dan Asia Tenggara. Walau bagaimanapun, kesan *C. excavata* pada luka masih tidak diketahui. Dalam kajian ini, penyembuhan luka dan kesan gastroprotective ekstrak daun ditentukan. Pemeringkatan berturutan daun menggunakan eter petroleum (PT) diikuti dengan kloroform (CH), etil asetat (EA), dan metanol (MOH) menunjukkan bahawa fraksi MOH memiliki jumlah kandungan fenolik tertinggi dengan nilai 522.0 ± 11.6 mgGAE/g dan aktiviti antioksidan yang paling tinggi. Nilai potensi antioksidan secara pengurangan ferik (FRAP) adalah 4649 ± 11.3 μmol Fe^{2+}/mg, manakala aktiviti memerangkap radikal menggunakan 2,2-Diphenyl-1-picrylhydrazyl (DPPH) adalah 86.89 ± 1.4% dengan IC_{50} yang rendah pada 23 μg / mL. MOH tidak toksik terhadap keratinocyte manusia (HaCaT), makrofaj murin (RAW 264.7), dan sel Vero apabila ditentukan oleh 3-(4,5-dimethylthiazol-2-YL)-2,5-diphenyltetrazolium bromida (MTT). Selanjutnya, kajian ketoksikan akut menunjukkan rawatan dengan fraksi MOH walaupun pada dos yang tinggi, 5000 mg / kg berat badan tidak menyebabkan kematian. Di samping itu, fraksi MOH tidak menjejaskan parameter hematologi atau berat relatif hati atau buah pinggang. Pra-rawatan dengan fraksi MOH merangsang percambahan dan pemindahan ketara sel fibroblast ($p < 0.05$) pada 24 dan 48 jam selepas rawatan dengan dibuktikan oleh penutupan kawasan luka dengan cepat berbanding dengan sel kawalan yang tidak dirawat. Rawatan topikal luka pada tikus Sprague-Dawley dengan 50, 100 dan 200 mg/mL fraksi MOH setiap hari selama 15 hari telah di dapati berkesan dalam merangsang penyembuhan luka dengan mengurangkan saiz luka, meningkatkan pengecutan luka, meningkatkan pertumbuhan semula tisu dan granulasi tisu, yang lebih signifikan ($P < 0.05$) berbanding tikus kawalan yang tidak dirawat. Penyembuhan luka kesan dari fraksi MOH adalah disebabkan oleh yang peningkatan signifikan ($P < 0.05$) perubahan pada faktor pertumbuhan beta (TGF-β1), faktor pertumbuhan vaskular endothelia (VEGF), superoksida dismutase (SOD), katalase (CAT), glutathione peroksidase (GPx) dan interleukin 10 (IL-10), manakala penurunan yang signifikan ($P < 0.05$) dalam cyclooxygenase-2 (COX-2), faktor tumor nekrosis alfa (TNF-α), interleukin 6 (IL-6) dan peroksidaan lipid (LPO). Penilaian histologi menggunakan haematoksilin dan eosin dan kaedah noda Masson trichome menunjukkan bahawa kulit yang sembuh selepas dirawat dengan fraksi MOH mempunyai kurang parut dan percambahan fibroblast yang lebih tinggi, pemendapan kolagen, dan angiogenesis berbanding kawalan yang tidak dirawat. Selain itu, ungkapan BAX, COX-2, leukemia B-sel / limfoma-2 (BCL2) gen ditentukan oleh teknologi awal menggunakan RT-PCR. Fraksi MOH menyebabkan apoptosis disahkan dengan penurunan regulasi BAX dan peningkatan regulasi BCL-2 gen. Untuk menentukan kesan gastroprotective fraksi MOH, ulser gastrik yang diinduksi etanol secara oral dan ikatan pilorus model tikus telah digunakan. Fraksi MOH menunjukkan kesan anti-perembesan asid dalam perut yang signifikan ($P < 0.05$). Pra-rawatan dengan fraksi MOH meningkatkan SOD, GPx dan CAT dengan ketara ($P < 0.05$) dan penurunan aktiviti LPO. Terdapat peningkatan ungkapan HSP70 dan TGF-protein β1 dan penurunan ungkapan protein Bax dalam tisu perut tikus. Fraksi metanol juga mengecilkan kawasan ulser, menurunkan penyusupan leukosit dan lapisan edema submucosal perut, meningkatkan pH dan merendahkan jumlah keasidan jus gastrik. Fraksi MOH telah diprofil oleh LCMS / MS dan ditunjukkan mengandungi konjugat myrisetin glucosida, quercetin-rhamnose-hekose, konjugat kaempferol, asid fenolik, flavonoid, furocoumarin, dan 8-geranyloxy psoralen. Kajian ini menunjukkan bahawa fraksi MOH memudahkan penyembuhan luka dan mempamerkan kesan perlindungan daripada ulser gastrik dengan merangsang aktiviti enzim antioksidan tisu, dengan itu

menghalang kecederaan tisu akibat radikal bebas dan merangsang pengantara keradangan, serta mengawal selia apoptosis.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
°C	:	Degree Celsius
ALP	:	Alkaline phosphatase
Alt	:	Alanine amino transferase
ANOVA	:	Analysis of variance
AST	:	Aspartate amino transferase
ATCC	:	American type culture collection
Bad	:	BCL-2 associated death protein
Bak	:	BCL-2 homologous antagonist killer
Bax	:	BCL-2 associated X protein
CAT	:	Catalase
CH	:	Chloroform
DAB	:	Diaminobenzidine
DMSO	:	Dimethyl sulfoxide
DMEM	:	Dulbecco's modified eagle medium
DPPH	:	2, 2-Diphenyl-1-picrylhydrazyl
ECM	:	Extracellular matrix
EA	:	Ethyl acetate
FRAP	:	Ferric reducing antioxidant power
FeIITPTZ	:	Ferrous tripyridyl triazine
FeIIITPTZ	:	Ferritri pyridyl triazine
GPx	:	Glutathione peroxide
H&E	:	Haematoxylin and Eosin
H. pylori	:	*Helicobacter pylori*

HD	:	High dose
HSP	:	Heat shock protein
IL-6	:	Interleukin 6
IL-10	:	Interleukin 10
LC-MS	:	Liquid chromatography-mass spectrometry
LPO	:	Lipid peroxidation
Mg	:	Milligram
MFCE	:	Methanolic fraction *Clausena excavata*
µg	:	Microgram
Ml	:	Milliliter
µg	:	Microgram
µL	:	Microlitter
µmol	:	Micromole
µL	:	Microliter
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide
MOH	:	Methanol
PAS	:	Periodic acid Schiff
PBS	:	Phosphate buffer saline
PDGF	:	Platelet-derived growth factor
PGE2	:	Prostaglandin E2
PPIs	:	Proton pump inhibitors
PT	:	Petroleum ether
P value	:	Level of significance
S.D	:	Standard division
SD rats	:	Sprague dawley rats
SOD	:	Superoxide dismutase

TB	:	Total bilirubin
TGF-β	:	Transforming growth factor-beta
TNF.α	:	Tumor necrosis factor-alpha
TPTZ	:	Pyridyl triazine
UMMC	:	University Malaya, Medical Center
VEGF	:	Vascular endothelial growth factor
VWF	:	Von willebrand factor
Wt	:	Weight

CHAPTER 1: INTRODUCTION

1.1.Wounds

The Wound Healing Society defines wound as a kind of break in the normal anatomic structure and functions of the skin against the exterior environment. Generally skin wounds are generated from chemical, physical and microbial offensive harm to the tissue (Rezaei et al., 2015). Wounds may be classified as closed (e.g. ruptures and sprains) or open depending on physical damages that result in an opening of the skin (e.g. ballistic, hernias and excised or surgical wounds). Wound can also be classified as being either acute or chronic and as partial thickness or full-thickness wounds. Acute wounds are defined as wounds that heal in an expected time while, chronic wound are those wounds that are heal slowly. This usually occurs in compromised patients who have an underlying pathology conditions such as malignancies or diabetes (Boateng et al., 2008). Partial thickness wounds involve the epidermis and may or may not involve the dermis. They have mostly healed first with inflammatory sensation, then re-epithelialization. However, in full thickness wounds, healing begins after injury initiates a series of combination of cellular-molecular events that occur in proper sequential and overlapping phases within a specified period in the healthy host (Guo & DiPietro, 2010). Wound healing is a biological process that attempts to cure injuries that occurred in the defensive barrier tissue. A series of cellular and molecular reactions occur consequently, prompting the response of inflammation, in which the cells in the dermis layers begin to produce more collagen to regenerate the epithelial tissues (Guo & DiPietro, 2010).

Singer and Clark (1999) specified that wound repair is a complex immune-mediatedphysiological processes, which involve a series of well-organized events

including hemostasis, inflammation, granulation tissue formation, neovascularization, degradation and synthesis of collagen, epithelialization, and wound remission, each of which is guided by the action of net cells of blood, growth factors and pro-inflammatory response cytokines, which are responsible to revitalization the wounded tissue to as near normal function as possible. Undoubtedly, transforming growth factor beta (TGF-β) and cytokines participate in the contraction of wound, while TGF-β, tumor necrosis alpha (TNF-α) and vascular endothelial growth factor are all important enhancers of cell proliferation, migration, chemotaxis and angiogenesis in the wound healing process (Jing et al., 2011; Huebener & Schwabe, 2012).

It has been well established that excess ROS results in the killing of fibroblasts, resulting in the loss of skin flexibility. Thus, antioxidants seem to be efficient in the treatment and management of wounds, through eliminating products of inflammation (Park & You, 2016). Because of this, researchers have tried to find medications from natural sources which have high antioxidant defense potentials in order to cure wounds (Ipek et al., 2012).

Cells participate in each phase of the wound healing process, and expire through one of three probable ways, which are apoptosis, necrosis and emigration. BCL2 family proteins either promote or prohibit apoptosis. The pro-apoptotic proteins involve BAX, Bad and Bak, whereas B-cell lymphoma 2 is anti-apoptotic proteins. Apoptosis plays a pivotal role in the synchronization of speedily altering cell residents, which is complicated in all tissue healing. For a massive common wound, controls of proliferation causes an acceleration of the wound closure. Sometimes the equilibrium between the decrease and increase in the number of cells forfeits its equilibrium and causes pathologic tissue healing (Walaa et al., 2014).

Wound healing starts immediately after injury has occurred, but the mechanism, speed of healing, and the eventual nature of the regenerated tissue depends on the type

of damage. In wound healing process, the wound environment alter with changing individual health status (Hardy, 1989). Activation of complicated net cells of blood, tissues, growth factors and cytokines cause increased cellular activity that causes rising metabolic requests for nutrients. Deficiencies in nutrition could hold up repairing of the wound. Many nutrition agents which might enhance wound healing processes time such as, vitamin A, required for epithelial and bone formation and, vitamin C are essential for collagen synthesis and functioning of the immune system (David & Heather, 2000).

Burns and poor wound healing are major medical problems in developing countries. The management of chronic wounds is a major problem because of poor hygiene conditions that lead to bacterial infections, resulting in high cost of therapy and presence of undesirable side effects (Umachigi et al., 2007). Even though a handful of topical antibiotic creams are available over the counter, most of them are either expensive to afford, prone to misuse resulting in antibiotic resistance or have a low antioxidant activity and a narrow safety margin. Thus, the evaluation of natural compounds with potent antioxidant activities as potential wound healing agents is paramount in order to discover newer effective wound healing agents. Therefore, this study is designed to throw an insight on the wound healing activity of *Clausena excavata* in experimental rat model, with the hope that it may help reduce the cost of hospitalization and reduction in the incidence of amputation arising from wound complications. The study may also lead to a better understanding of the influence of natural products on wounds, which forms a platform for further studies that open up possibility of finding alternative therapy for wound management.

1.2. Peptic Ulcer

Peptic ulcer is the most widespread digestive condition that can be defined as mucosal incision, which may spread throughout the muscular layers into submucosa layers, as a result of gastric acid and pepsin secretion. In some cases, the condition can be severe and may lead to many complications such as malignancy. The peptic ulcer term comprises both gastric ulcer and duodenal ulcer (Ramakrishnan & Salinas, 2007). Clinical symptoms and complications include; nausea, vomiting, severe epigastric sore, cardiac burning, and loss of appetite (Ramakrishnan & Salinas, 2007).

Gastric and duodenal ulcers occur because of an imbalance between destructive and cyto-protective factors. The destructive factors include endogenous and exogenous factors such as pepsin, excess hydrochloric acid and reflux of alkaline duodenal contents containing pancreatic enzymes with bile (Wormsley, 1972; Vetro et al., 2002). Smoking, stressful lifestyle, alcohol beverage, heavy diet, coffee, and in some cases, a positive family history (Morsy & El-Sheikh, 2011), and over dose drugs, especially ibuprofen, aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) are aggressive tools which have serious exogenous mucosal excitations that can cause mucosal damage as well as *Helicobacter pylori* infection (Ko & Cho, 2000; Weil et al., 2000). Conversely, the gastroprotective factors against ulcer formation are gastric mucus-bicarbonate wall function excreted through superficial epithelial cells; prostaglandins (PGE2), gastric mucosal microcirculation and endogenous enzymatic antioxidants activity to reduce mucosal damage caused by inflammatory cells are considered important in maintaining gastric mucosal safety, as well as some other growth factors such as TGF-β, which plays an important role where it regulates epithelial cell proliferation, inflammation and tissue repair (Grisham et al., 1987; Wallace & Granger, 1996; Abdel-Salam et al., 2001).

The body is usually under an active balance between free radical generation and endogenous scavenging enzyme systems, such as superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GSH). All these molecules have a central antioxidant function in scavenging reactive oxygen species (ROS) with a redox based mechanism (Brambilla et al., 2008). The existence of drug metabolism causes ROS generation to overpower the cellular antioxidant defense which leads to oxidative stress (Verma et al., 2013).

Gastric ulcer is a global problem in developed countries and can impair community health and socio-economic status of affected countries (Zissin, 2008). Anti-secretory medications frequently used to treat gastric ulcer include; antacids like aluminum hydroxide, histamine H2-antagonists such cimetidine and ranitidine and proton pump inhibitors (PPI) such as omeprazole, all have undesirable effects, such as diarrhea, hypercalcemia (leading to kidney failure), kidney stones, cardiac arrhythmias and costly for populations (Eid et al., 2010). Besides, none of the reciprocal antiulcer agents can achieve healing completely (Borrelli & Izzo, 2000). Therefore, researchers have a growing interest in searching for alternate medicines which are safe and have anti-gastric ulcer effects without any adverse effect. Nowadays, potential and promising medicinal plants and their natural products are widely used for medicinal purposes as alternate medicines in treatment of ulcer. Traditional medicine is growing globally and eastern world of developing countries consider natural flora as the only choice of treatment, including Malaysia. (Al Mofleh et al., 2008). Medicinal plants are the main source of natural active compounds such as alkaloid, phenolic compounds, terpinoid and tannins that are involved in direct or indirect protection of the body from a variety of diseases, display antiulcer activities and may influence one or more phases of wound healing because of their influence on growth stimulation and antioxidant effects (de Souza Ameida et al., 2011; Ponrasu et al., 2014). Malaysian plants are used traditionally

to treat various diseases and reported to have potent anti-ulcer activities such as *Phylanthus minus, Curcuma xanthorrhiza, Momordica charantia* (Wasman et al., 2012), which have bioactive capacity and gastroprotective properties in the gastric mucosa. Some of the plants that have been tested for wound healing potentials include *Acalypha langiana* (Perez and Vargas, 2006), *Radix astragalis* and *Radix rehmannia* (Lau et al., 2009), *Rosmanis officinalis* L (Abu-Al-Basal., 2010), *Curcuma longa (*Sidhu et al., 1999), *Sparassia crispa* (Kwon et al., 2009), *Hylocereus undatus* (Perez et al., 2005), *Momordica charantia* (Teoh et al., 2009), *Lithospermum erythrorhison* (Fujita et al., 2003), *Aloe vera* (Atiba et al., 2011).

Evaluation of various plant products according to their traditional uses and medicinal value, based on their therapeutic efficacy leads to the discovery of newer and cost effective drugs for treating various ailments. This forms the basis for this study, which is to develop newer therapeutic agent from *C. excavata,* which may be useful in facilitating wound healing and gastro-protection in animal models. In this research project, *C. excavata* was chosen because it is one of the natural products of interest in the field of pharmacology, and based on previous studies, it has good antioxidant, antimicrobial and immunomodulatory activities. (Blumenthal et al., 2000). Furthermore, besides its traditional use in wound and stomach-ach (Zheng et al., 2013), there is no documented scientific evidence on other potentials of the plant. Hence, it is important to investigate the wound healing and anti-ulcer effect of *C. excavata* against gastric ulcer in rat model and wound healing in order to provide a valuable contribution to the knowledge database of phytomedicines.

Some researches have proved toxicity effects of few Malaysian plants in experimental animals. Toxicity evaluation of every medicinal plant is essential and vital

procedure for any future commercial desire in product therefore; acute toxicity of *C. excavata* in *vivo* was also evaluated to determine the safe dose that can be used.

1.3. Objectives of this study

The present study was conceptualized to evaluate the wound healing efficacy of topical administration of *C. excavata* using *in vivo* experimental wound model and gastroprotective effect by modulating inflammatory response.

1.3.1. Specific objectives:

1. To evaluate the antioxidant, antibacterial and cytotoxicity fractions
2. To evaluate its *in vitro* wound healing effects through proliferation of *C. excavata* and migration of fibroblast cells using wound scratch test assay.
3. To determine the phytochemical components of methanol fraction of *C. excavata* by LCMS (Liquid chromatography–mass spectrometry.
4. To determine the acute toxicity of *C. excavata* methanol fraction in rats.
5. To evaluate the mechanisms of wound healing of methanolic fraction of *C. excavata* in animal model and estimate the changes in apoptosis and inflammatory releated genes (BAX, BCL2 and COX-2) in wound healing model.
6. To determine the effect of *C. excavata* on the gastric acid output in vivo and to evaluate its gastroprotective mechanism in gastric ulcers-induced animals.

CHAPTER 2: LITERATURE REVIEW

2.1. Wound and wound healing

A wound defined as any damage involving division of tissue or rupture of an integument or mucous membrane. The pathological definition of a wound is a sharp injury that damages the dermis of the skin (Boateng et al., 2008). An open wound is when the skin is cut or ruptured while a closed wound occurs when blunt force trauma is inflicted to the skin resulting in contusion. Wounds can be classified according to the cause of injury: mechanical, burns or chemical (Aljady, 2003).

Wound healing is defined as a process that leads to restoration of tissue integrity, functions of injured skin, and replacement of destroyed tissues by living cells (Watson, 2003). Dissimilar wounds include incomplete thickness incision wound and full thickness excisional wound, which vary in the degree of healing (Monaco & Lawrence, 2003). Wounds are accompanied by pain, reddening and edema, which are the classical symptoms of inflammation. These symptoms are caused by the release of eicosanoids, prostaglandins, leukotrienes, and reactive oxygen species (ROS). Wound healing can also be retarded by microbial infections. The production of reactive oxygen species (ROS) produced at the site of the wounds is one of the defense mechanisms against infections. However, since free radicals can damage tissue cells, their production in high quantities also hampers wound healing (Ilango & Chitra, 2010).

2.2. Phases of wound healing

Wound healing comprises a series of events that occur in structured manner. The events in wound healing ultimately culminate in the final repair of injured tissues. Wound healing is characterized by five phases, namely hemostasis, inflammation, proliferation, protein synthesis and wound contraction, and remodeling (David & Heather, 2000; Barrientos et al., 2008) (Figure 2.1).

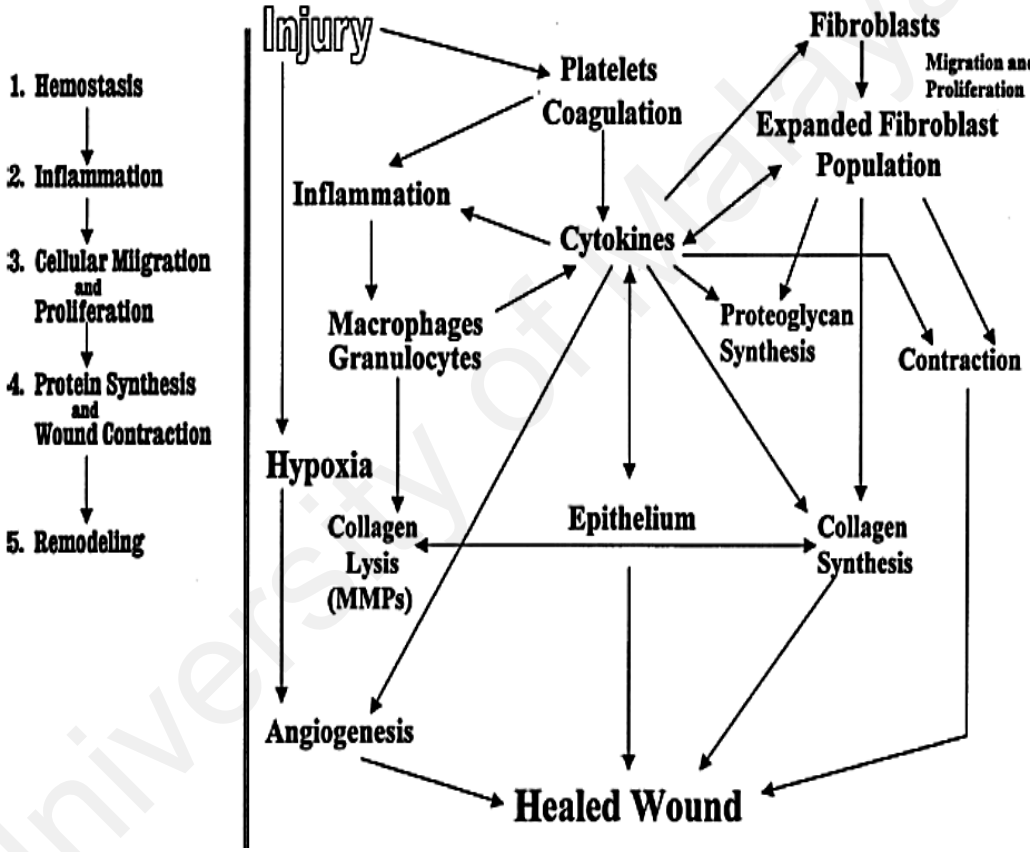

Figure 2.1: Acute wound healing cascade. The propulsion of acute wound healing from hemostasis to the final phases of remodeling is dependent on a complex interaction between several wound-healing events. Cytokines play a fundamental role in wound healing and act as a central signal for various cell types in healing.
Adapted from (Monaco & Lawrence, 2003).

2.2.1. Hemostasis

Trauma to tissues cause vascular damage and initiates molecular and cellular response that promote hemostasis. Blood vessel damages allow circulating platelets to come into contact with the exposed collagen and other elements of the extravascular matrix. If there are bacterial infections, chemical signals released by the organism act as chemo attractants to neutrophils and these cells enter the wound site and begin the critical task of removing foreign materials, bacteria, and damaged tissues (Borkow et al., 2008). Achieving hemostasis is vital for the healing process to commence. Vasoconstriction, platelet aggregation, and fibrin deposition are primary contributors of hemostasis. The coagulation cascade is also initiated resulting in formation of blood clot composed of fibrin networks containing aggregated platelets deposited at surrounding tissues (Lawrence, 1998).

2.2.1.1. Vasoconstriction

Release of vasoactive amines by the cells of injured tissues contributes to the initiation of vasoconstriction. Simultaneously, in tissue injury the sympathetic nervous system liberates norepinephrine that functions to constrict the capillaries. In tissue injuries, prostaglandins such as thromboxane are also secreted from the injured cells and these cytokines are major contributors to vasoconstriction (Monaco & Lawrence, 2003).

2.2.1.2. Platelet aggregation

Platelets contain secretory granules, primarily α-granules, dense bodies, and lysosomes. In tissue injuries, platelets become activated upon coming in contact with exposed extravascular matrix and liberate substances from their granules (Cines et al., 1998; Borkow et al., 2008). Among these substances are a variety of proteinaceous and immunomodulatory agents from α-granules. These substances include adhesive proteins (Von Willebrand factor, fibrinogen, fibronectin, and thrombospondin), platelet-derived

growth factor (PDGF), fibroblast growth factor-2 (FGF-2), transforming growth factors α and β (TGF-α & TGF-β), epidermal growth factors (EGFs), and endothelial cell growth factors. Calcium, serotonin, ADP, and ATP found in the dense bodies are also mediators that facilitate healing (Griendling et al., 2000).

2.2.1.3. Fibrin and the coagulation cascades

The coagulation cascade comprises of the intrinsic, extrinsic, and common pathways. Thrombin that is produced through the activation of these pathways catalyzes the formation of fibrin from fibrinogen. The fibrin network consists of protein polymers that anchors platelet plugs forming temporary matrix of cells and proteins that protect wound and facilitate healing. The binding of fibrin to the aggregated platelets occurs through several cell surface receptors including fibronectin receptors (Dahlbäck, 2005).

2.2.2. Inflammation

Inflammation is an essential component of tissue repair and is one of the prerequisites in healing. The cardinal signs of inflammation are erythema, heat, edema, pain, and loss of function. Erythema and heat develop in inflammation soon after injury as a result of vasodilatation, while the pain sensation is due to the accumulation of fluid in the injury causing edema (Monaco & Lawrence, 2003). The inflammatory events occur within the first six to eight hours post-injury with heavy infiltration of polymorph nuclear leukocytes (PMNs) into the tissue (Figure 2.2). Among functions of PMNs include phagocytic removal of bacteria in infections and dead cells from tissue injury. This role of PMNs in inflammation is fundamental to tissue repair (Li et al., 2007; Merchandetti & Cohen, 2008).

Inflammation occurs in two phases; vascular and cellular. In the vascular phase, blood flow and blood vessel permeability in the injured tissue increase. Early in the

vascular phase, there is vasoconstriction leading to temporary blanching of wounds. This process which is initiated by circulating vasoconstrictors serves to limit the hemorrhage and concentrates healing factor within the area of the wound (Gabrie, 2015). Eventually vasodilatation ensured after 10-15 min of wound infliction. During this phase, there is an increased diffusion of plasma out of blood vessels into the extravascular spaces (Monaco & Lawrence, 2003). With vasodilation, fluid flows into the injured area and become inflammatory exudates that contain plasma proteins, antibodies, erythrocytes, leucocytes, and platelets (Majewska & Gendaszewska-Darmach, 2011). The switch from vasoconstriction to vasodilation is induced by endothelial and mast cell derived factors such as a group of arachidonic acid metabolites called leukotrienes. Leukotriene (LT) B4 is also a potent chemotactic factor for neutrophils, whereas LT4, LTD4 and LTE4 cause vasoconstriction and increased vascular permeability. Prostaglandins, PGI2, PGD2, PGE2, and PGF2α, and histamine contribute to vasodilation (Noli & Miolo, 2001). These effects are enhanced by kinin produced from kinogen. Kinin besides being a chemotactic factor also enhances phagocytosis and causes pain by stimulating sensory nerve endings (Frey et al., 2006).

Cellular reaction of the inflammatory phase occurs within hours of injury and this is characterized by increased in leukocyte infiltration, particularly neutrophils and monocytes, into the wound area. Since neutrophils are the predominant circulating leukocytes, they are the first cell type to response. Therefore, in the first 48 hours of the inflammatory response, neutrophils predominate (Li et al., 2007). The most important cell that controls inflammatory reaction is the macrophage. Like neutrophils, macrophages can phagocytize, digest, and kill pathogenic organisms and scavenge tissue debris and destroy any residual cells. Macrophages also release enzymes and biologically active oxygen intermediates that initiate angiogenesis and the formation of granulation tissues (Lewis et al., 1999; White et al., 2004). Another important role of

macrophages is in the release of cytokines including fibronectin, fibroblast growth factor, and vascular endothelial growth factor. The cytokines attract endothelial cells to the wound, stimulate their proliferation, and promote angiogenesis. Macrophages thus act like factories for growth factor production and play an important roles in the transformation of inflammation into repair of tissue (Bosco et al., 2008).

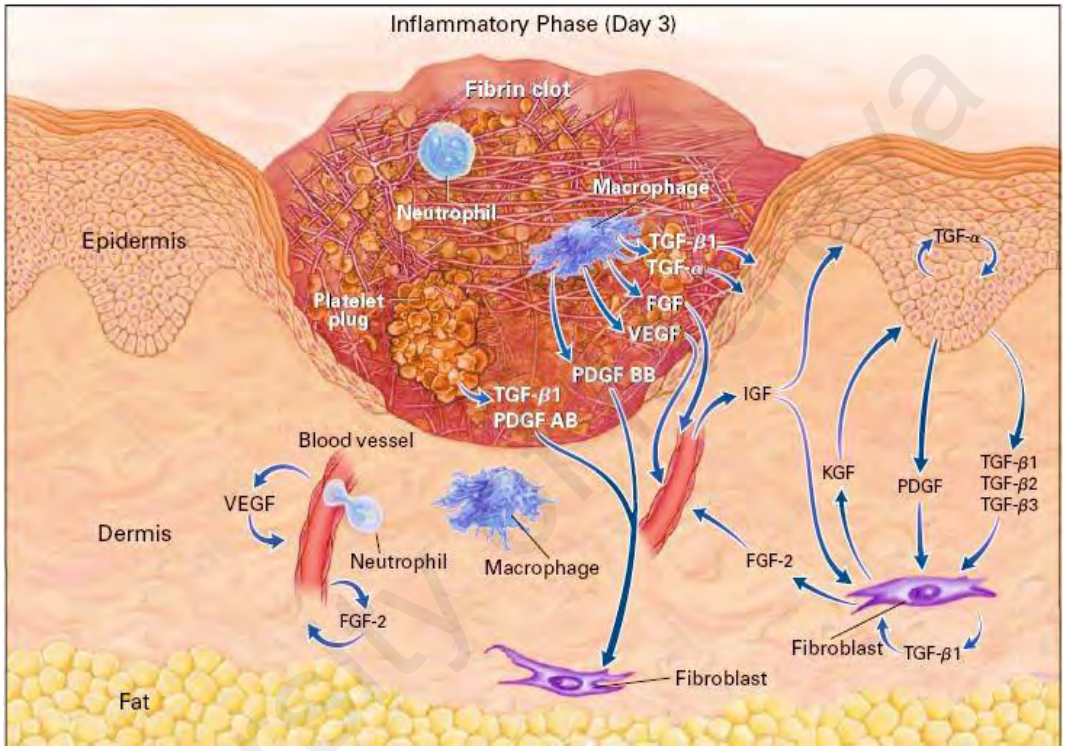

Figure 2.2: Cutaneous wound healing at day 3 after injury. Growth factors are thought to be necessary for cell movement and wound healing. In this stage there is expression of TGF-β1, TGF-β2 and TGF-β3. TGF-α, fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), insulin like growth factor (IGF) and keratinocyte growth factor (KGF). Adapted from (Singer & Clark, 1999).

2.2.3. Proliferation

This phase begins with the completion of the inflammatory phase, usually between days 4-14 after injury (Figure 2.3). The proliferative phase is characterized by epithelialization, angiogenesis, granulation tissue formation, and collagen deposition (Diegelmann & Evans, 2004). Endothelial cells produce vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF). In tissue injury, intact epithelial cells within the margin of the wound, with the assistance of the growth factors, begin to reproduce within hours of injury. However, the presence of necrotic or sloughed tissue impairs epithelialization. In this case, healing can only be efficient after damaged tissues are physically removed by debridement (Davies et al., 2005; Ramundo & Gray, 2008). Modeling and establishing of new blood vessels is critical in wound healing and this occurs simultaneously through all stages of the reparative process (Pierce et al., 1991; Takeshita et al., 1994).

Granulation tissue is new connective tissue with minute vascular supply which forms on wound surface during healing. Formation of granulation tissue involves participation of structural molecules such as fibronectin and collagen that provide a scaffold for the new tissue. Collagens, produced by fibroblasts, act as a foundation for the cellular matrix within the wound (Baum & Arpey, 2005; Diegelmann & Evans, 2004). The formation of collagen by fibroblasts is stimulated by numerous cytokines, PDGF, TGF-β and EGF. Strengthening of new tissue growth requires filling of the intercellular spaces, this is the role of glycosaminoglycans (GAG). The GAG ground substance combined with water and acts as lubricant to assist movement of collagen fibers (Barrientos et al., 2008).

The rate of synthesis of collagen is influenced by several factors including age, tension, pressure, and stress (Velnar et al., 2009). Among the role of collagen is as a

contractile element of the wound that cause the wound to contract and shrink. The contraction of the wound is the prerequisite for formation of the scar that eventually flattens into a thin white line (Mahmood et al., 2010). The strength of a scar depends on the quality and quantity of deposition of structural proteins during healing (Bullard et al., 1999). In fact, the protein content should be more than 50% for the scar tissue to heal properly (Diegelmann & Evans, 2004). Other components of the wound matrix include fibrin and fibronectin. The composition of the wound matrix changes with healing, fibrin and fibronectin gradually replaced by collagen and proteoglycans.

Open wounds for duration of two weeks or more takes longer to contract. The maximum rate of contraction is 0.75 mm per day, however this differs with anatomic site. The rate of contraction is also influenced by shape of the wound; square wound contracts more than circular wounds. Myofibroblasts found at the periphery of wounds is a cardinal feature of wound contraction. The myofibroblasts appear four to six days after the initial injury and remain present for 2 to 3 weeks (Porter, 2007). These cells are essential in wound healing because they act as the ‘‘motor’’ of wound contractions. It was suggest that TGF-β is also among cytokines participating in wound contraction (Huebener & Schwabe, 2012).

2.2.4. Remodeling

Tissue remodeling is the final stage of wound healing and it is also known as the maturation phase (Figure 2.4). This stage is most important because it also determines the type of scar that is formed. Scar remodeling involves aggregation, orientation and arrangement of collagen fibers (Diegelmann & Evans, 2004) and this dependents on the continuous collagen synthesis in the light of the collagen destruction. During healing, collagen is constantly degraded and replaced. Thus, the balance between synthesis and removal of collagen also determines the final form of the scar tissue (Atala et al., 2010).

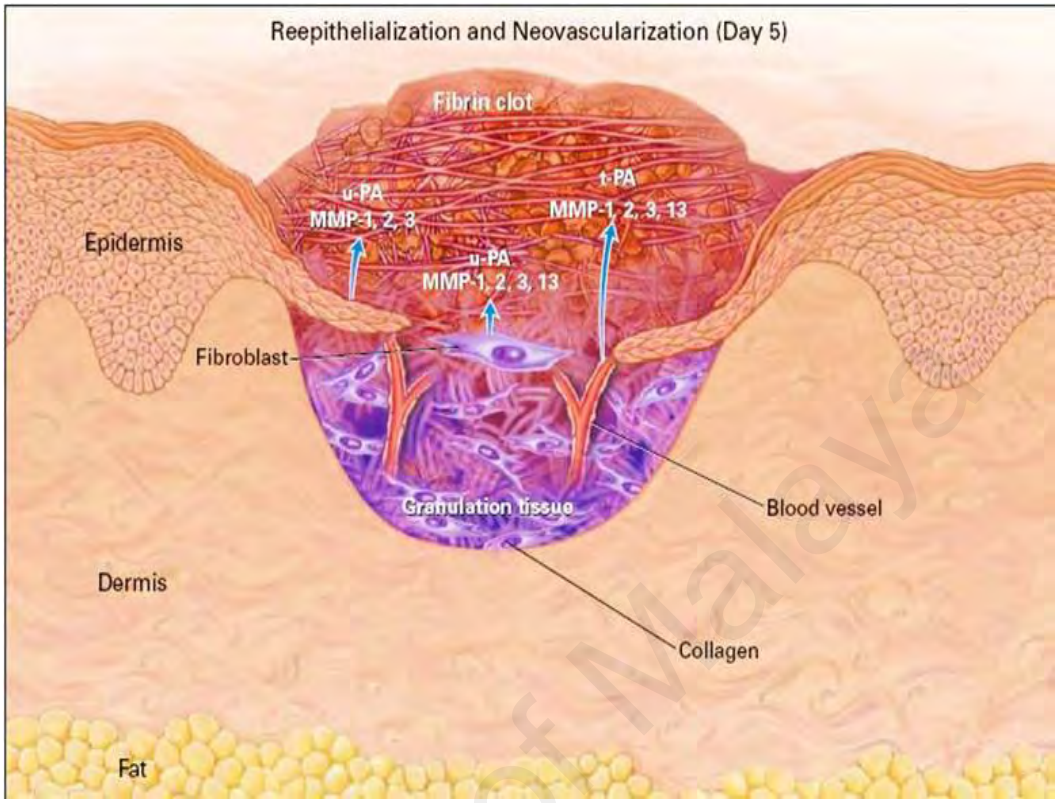

Figure 2.3: Cutaneous wound healing at day five after injury. Blood vessels have appeared to be sprouting into the fibrin clot as epidermal cells resurface the wound. Proteinases, thought to be important for cell movement. In this phase there is expression of urokinase type plasminogen activators (u-PA), matrix metalloproteinases1, 2, 3 and 13(MMP-1, 2, 3 and 13) and tissue plasminogen activator (t-PA).
Adapted from (Singer & Clark, 1999).

Matrix metalloproteinases (MMPs) play number of roles during wound repair, including removal of non-vital tissue, stimulation of blood vessel growth and keratinocyte migration, and connective tissue remodeling. The MMPs are implicated in the breakdown of collagen and facilitate angiogenesis during tissue remodeling. The modulation of action by MMPs is via the tissue inhibitors of metalloproteinase (TIMPs) (Brew et al., 2000; Gawronska-Kozak, 2011). The equilibrium between actions of

MMPs and TIMPs in the tissue is controlled by several cytokines (Haroon et al., 2000; Hayden et al., 2011).

Normal skin has a type 1: type III collagen ratio of 4:1. In wound healing, collagen increases in quantity reaching a maximum level by week three post-injury (Li et al., 2007; Rosch et al., 2002; Merchandetti & Cohen, 2008). The modifications during healing involve continuous degradation and synthesis of these collagen until the ratio for normal skin is achieved. At the final phase of wound healing, there is tissue remodeling, formation of new epithelium and scar tissue.

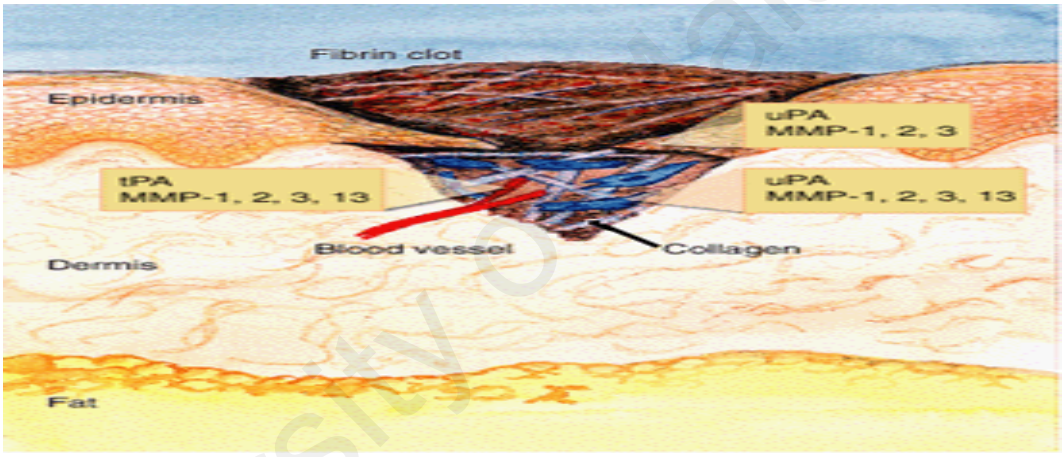

Figure 2.4: Cutaneous wound healing in remodeling phase. The remodeling phase of wound healing is cytokine-mediated. Degradation of febrile collagen and other matrix proteins is driven by serine proteases and MMPs under the control of the cytokine network. Granulation tissue forms below the epithelium and is composed of inflammatory cells, fibroblasts and newly formed and forming vessels.
Adapted from (Wahl, 2002).

2.3. Factors Affecting Wound Healing

Extrinsic and Intrinsic factors that may influence the rate of wound healing include infection, tissue oxygenation, nutrition, and age (Guo & Dipietro, 2010).

2.3.1. Infection

Wound infections are common in people of under developed and developing countries because of poor hygiene (Kumar et al., 2007). Presence of bacteria in the wound is detrimental to healing because endotoxins secreted by living bacteria in the wounded tissue inhibit the normal function of cells and tissues resulting in slow wound healing. However, inflammation, which is a normal event in wound healing, facilitates the removal of contaminating microorganisms. The inflammation can be prolonged in case of severe and persistent infections, where microbial clearance is incomplete. Bacteria and endotoxins in wound can lead to the prolonged elevation of pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF-α resulting in extended inflammatory phase. If the infection persists, the wound may become chronic and fail to heal. This prolonged inflammation also results to an increased level of matrix metalloproteases (MMPs) in the tissue. The net effect of these responses is a shift in protease balance and rapid degradation of growth factors (Guo & Dipietro, 2010).

2.3.2. Tissue Oxygenation

Tissue oxygenation is critical for wound healing, especially for the production of energy during cell and tissue regeneration. Among effects of tissue oxygenation are stimulation of angiogenesis, increased keratinocyte differentiation and migration, re epithelialization, enhanced fibroblast proliferation and collagen synthesis, and wound contraction (Bishop, 2008; Rodriguez et al., 2008). Oxygenation is associated with ROS

production by neutrophil and macrophages during phagocytosis. Reactive oxygen species are very effective in the destruction of bacteria.

Vascular disruption and high normal tissue oxygen consumption affect rate of wound healing. Impaired blood flow can occur in old age and diabetes resulting in tissue hypoxia. Although transient hypoxia activates wound healing, extended or prolonged hypoxia disrupts wound healing. In acute wounds, hypoxia serves as a signal for stimulation of wound healing through production of cytokines from macrophages, keratinocytes, and fibroblasts. These cytokines especially PDGF, TGF-β, VEGF, TNF-α, and endothelin-1 promote cell proliferation, migration and chemotaxis, and angiogenesis. For that reason the hyperbaric oxygen therapy is an option in the treatment of wounds and other tissue injuries (Guo & Dipietro, 2010).

2.3.3. Nutrition

Proteins are the chief building blocks in cell renewal, tissue growth and restoration. Proteins significantly influence all wound healing phases through collagen and elastic tissue formation, epidermal growth, keratinization, and initiation of immune response. In protein malnutrition, the skin becomes thin and wrinkled (Dryden et al., 2013). The deficiency of protein impairs immune responses and decrease leukocyte function and increase susceptibility to infection (Guo & Dipietro, 2010).

Several amino acids are particularly essential for the wound healing process. For example, arginine, a precursor to proline, is needed for collagen deposition, angiogenesis, and wound contraction (Campos et al., 2008). Glutamine is important for the proliferation of inflammatory cells and as a source of energy (Dryden et al., 2013) while cysteine and methionine are implicated in the production of collagen and connective tissue.

Glucose is the major source of fuel for the cell. Glucose deficiency affects cellular functions, and in wound healing the condition affects fibroblast proliferation (Han et al., 2004). In short, glucose deficiency or poor glucose utilization by the body retards wound healing.

Fatty acids are major structural and functional components cells and tissues. Fatty acids are required for tissue regeneration as an energy source and building blocks of tissues (Shingel et al., 2008). Polyunsaturated fatty acids (PUFA) alters pro-inflammatory cytokines. In fact, it was shown that eicosapentaenoic and decosahexaenoic PUFA increase pro-inflammatory cytokine production at the wound site (McDaniel et al., 2008). The fat-soluble vitamins A, D, E and K are essential micronutrients for the various metabolic roles of the organism. Vitamin A is essential for epithelial and bone tissue development, immune system function, and cellular differentiation. The vitamin while enhancing wound repair by facilitating early inflammatory phase also improve immune response, increase number of macrophages and monocytes infiltrating wounded tissue, supports epithelial cell differentiation, and modulates collagenase activity (Mackay & Miller, 2003). Vitamin C (ascorbic acid) is necessary for hydroxylation of proline and lysine during the synthesis of collagen. The vitamin cross-links and stabilizes the triple helix structure of collagen (Wild et al., 2010).

Inorganic nutrients are required in small quantities for the health of the organism. Zinc, for example is essential for wound healing. As a cofactor of several enzymes, zinc is involved in the biosynthesis of RNA, DNA, and proteins. Low tissue zinc will decrease wound closure and suppresses inflammatory process, predisposing the wound to infections. Zinc also effects response of keratinocytes, osteocytes, and leukocytes and its deficiency would retard wound healing (Rink & Gabriel, 2001; Wild et al., 2010).

Iron is a cofactor of prolyl and lysyl hydrolytic enzymes that are essential for the synthesis of collagen. As an integral component of hemoglobin, the oxygen transporter in circulation, iron deficiency results in anemia with consequential poor supply of oxygen to the tissues (Brown & Phillips, 2010). Thus, iron deficiency inhibits wound healing through poor tissue regeneration and persistent tissue hypoxia.

2.3.4. Age

Young adults and children generally heal well from wounds. Delayed wound healing in old subjects is associated with delayed inflammatory response, such as delayed T-cell infiltration into the wound area, abnormal chemokine production, and reduced macrophage phagocytic capacity (Swift et al., 2001). Wound contraction and re-epithelialization are slower in old people because of the low number of tissue fibroblasts and the consequentially reduction in collagen production. Among other geriatric factors that affect wound healing are peripheral ischemia, heart disease, and diabetes mellitus. Furthermore, reduced mobility in the elderly also increases risk of pressure to the affected tissues that may prolong the healing process (Gosain & Dipietro, 2004; Oriana et al., 2012).

2.4. Reactive Oxygen Species

Reactive oxygen species are pivotal regulators of normal healing of wound (Schwentker et al., 2002). Low levels of ROS are needed for protection against infections. ROS also act as cellular messengers in cell motility, action of cytokines, and angiogenesis (Rodriguez et al., 2008). An imbalance between antioxidants and free radicals results in oxidative stress. Free radicals can damage macromolecules and cause oxidative stress that contributes to the development of a number of diseases including alzheimer, diabetes, and neurodegeneration (Lee et al., 2007). Production of ROS can

be induced both in hypoxia and hyperoxia. During cellular metabolism, especially in neutrophils and macrophages, the reactive superoxide radical anions, under the catalytic action of superoxide dismutase, are rapidly converted to hydrogen peroxide (H_2O_2) and water. Hydrogen peroxide can cause serious cell injuries. Hydroxyl radicals, the product of H_2O_2 breakdown are highly aggressive factors that can cause oxidation and damage of cellular macromolecules. For this reason, the body rapidly detoxifies H_2O_2 through the action of catalase and peroxidases. In abnormal high production of ROS or when detoxification is ineffective, the accumulation of hydroxyl radicals causes serious cell and tissue damage (Steiling et al., 1999).

Reactive oxygen species are formed in high amounts at the site of the wound as a protection against invading bacteria (Srinivas et al., 2008). However, with increased in neutrophil infiltration, the production of ROS overpowers the anti-oxidation process of the injured tissue. At high concentrations, ROS can cause severe tissue injury or even neoplastic transformation and reduce the healing process via damaging cellular membranes, proteins, DNA, and lipids (Ipek et al., 2012).

2.5. The role of growth factors and cytokines in wound healing

Proinflammatory cytokines are expressed during inflammatory phase of wound healing process. These proinflammatory cytokines include IL-1β, IL-6, and TNF α. In chronic wounds, the profile of these cytokines and inflammatory responses are altered in such a way that the levels of these cytokines decrease as the chronic wound begins to heal, indicating a significant correlation between non-healing wounds and increased levels of pro-inflammatory cytokines. Chronic wound fluid containing these cytokines has also been shown to inhibit growth and induce morphological changes in normal skin fibroblasts (Mendez *et al*., 1999; Enoch and Price, 2004). The altered inflammatory repair process is associated with a high production of reactive oxygen species which

cause high levels of oxidative stress when compared to the normal healing process. Macrophage activation is decreased for the release of cytokines and growth factors to recruit fibroblasts, keratinocytes and endothelial cells, causing an altered inflammatory response. Likewise, the lymphocyte infiltration in chronic wounds is also altered.

2.6. Wound care

The main aim in wound treatment is to provide a conducive environment for reestablishment of normal tissue structure. In wound treatment, it is necessary to remove excessive exudates but not to a point that it causes wound to dry. It is imperative that wound infection is prevented to allow for rapid healing, preservation of tissue structure and function (Fox et al., 1985). Since granulation tissue formation is one of the stages in healing, any treatment that is instituted must not cause damage to developing granulation tissue.

2.7. Ethnobotanical Studies on Wound Healing

The current trend in drug development is in the discovery of compounds from natural products. There seems to be a growing list of natural products claimed to be efficacious in the treatment of wounds (Table 2.1). The products were shown to have several properties that could facilitate wound healing including anti-inflammatory, stimulation of wound contraction and re-epithelialization, and anti-microorganisms.

Plants have long been the source of useful products for humans. In Eastern and Southern Asia, traditional medicine is most preferred for treatment of various ailments (Gupta & Sharma, 2006). In these countries, traditional healers sought for treatments (Kongkathip *et al*., 2002) using traditional therapeutic compounds and regimes that are based on indigenous knowledge gained from ancestral experiences (Lewis & Elvin-Lewis, 1995).

Malaysia's rainforest contains a large collection of plant species with unexplored medicinal properties. Several plants have been evaluated and their medicinal properties identified (Alam et al., 2009). In fact, many modern drugs in the market today originated from natural products. Because of the potential of natural resources, particularly plants, as a source of therapeutic compounds, ethno pharmacology has become rapid expanding research area. Among common diseases targeted in the discovery of innovative compounds from natural resources are cancers, wounds, gastrointestinal and skin disorders (Yadav et al., 2011; Sasidharan et al., 2010). Although, several plant products are purported to be effect for these diseases, their efficacies have not been scientifically verified or documented.

In one study in rats, it was shown that the ethanolic extract of *Mallotus philippinensis* Muell fruit has potent wound healing capacity (Gangwar et al., 2015). The study showed that the extract enhanced wound contraction, decreased epithelialization period and scar area, enhanced antioxidants and connective tissue markers. Other extracts that showed wound healing properties are those of *Plantain banana* (Agarwal et al., 2009) and root tubers of *Curculigo orchioides* (Singh et al., 2014). The root tubers of *C. orchioides* significantly increased the level of superoxide dismutase, nitric oxide and decreased lipid peroxidation in granuloma tissue of diabetic mice. *Morinda citrifolia* leaf extract (Rasal et al., 2008), *Limonia acidissima* Linn (Rutaceae) (Ilango & Chitra, 2010) fruit pulp significantly increase contraction rate, tensile strength, granuloma breaking strength, collagen content, dry granuloma weight, hydroxyproline contents, and decrease period of epithelialization in wounds. These plants are potent sources of antioxidative phenolic compounds responsible for wound healing effects.

Table 2.1: Herbal medicine in wound care.

Herbal Medicine	Properties
Aspilia africana	Hemostatic properties on wounds Inhibits growth of microbial organisms Accelerates wound healing Treatment of rheumatic pain, bee & scorpion stings Clears corneal opacities & remove foreign bodies from eyes
Bridelia ferruginea, *Parkia biglobosa Jacq* *Elaeis guineensis* leaf extract	Increase proliferation of dermal fibroblasts Improve tissue regeneration
Cedrus libani, *Abies cilicica subsp. Cilicica* *carapa guianensis* leaf	Improve wound healing and anti-inflammatory properties Increase rate of wound contraction, skin breaking strength and hydroxyproline content
Combination of Yasha bhasma, Shoea robusta and flax seed oil,	Increase wound contraction, higher collagen content & better skin breaking strength
Hippophae rhamniodes L. *Carica papaya* latex	Improve wound healing Increase wound contraction & epithelialization rate
Methanol extract of *Heliotropium indicumLinn.*leaf	Improve wound healing rate
Rafflesia hasseltii bud & flower extracts	Improve wound healing rate and wound contraction
Melaleuca alternifolia	Anti-microbial, anti-septic, anti inflammatory, anti-fungal, & anti-viral properties

Adapted from Dorai, (2012).

2.8. Gastric ulcer

Gastric ulcer, a necrotic lesion of the gastric mucosa (Figure 2.5), is one of the most widely distributed chronic diseases affecting at least 10% of the population in the Western (Barkun & Leontiadis, 2010). In Asia and the South Pacific, gastric ulcer has been diagnosed in 11.5% of the population (Scott et al., 2013). In gastric ulcers, the mucosal layers of the stomach under the influence of various exogenous and endogenous factors are stretched causing disruption of the surface of stomach lining. Damage to the gastric mucosal is usually accompanied by increased acid secretion and pepsin production that enhance damage. The disease manifest as gastric and cardiac pain, weight loss, nausea, vomiting, hematemesis, and melena (Ramakrishnan & Salinas, 2007).

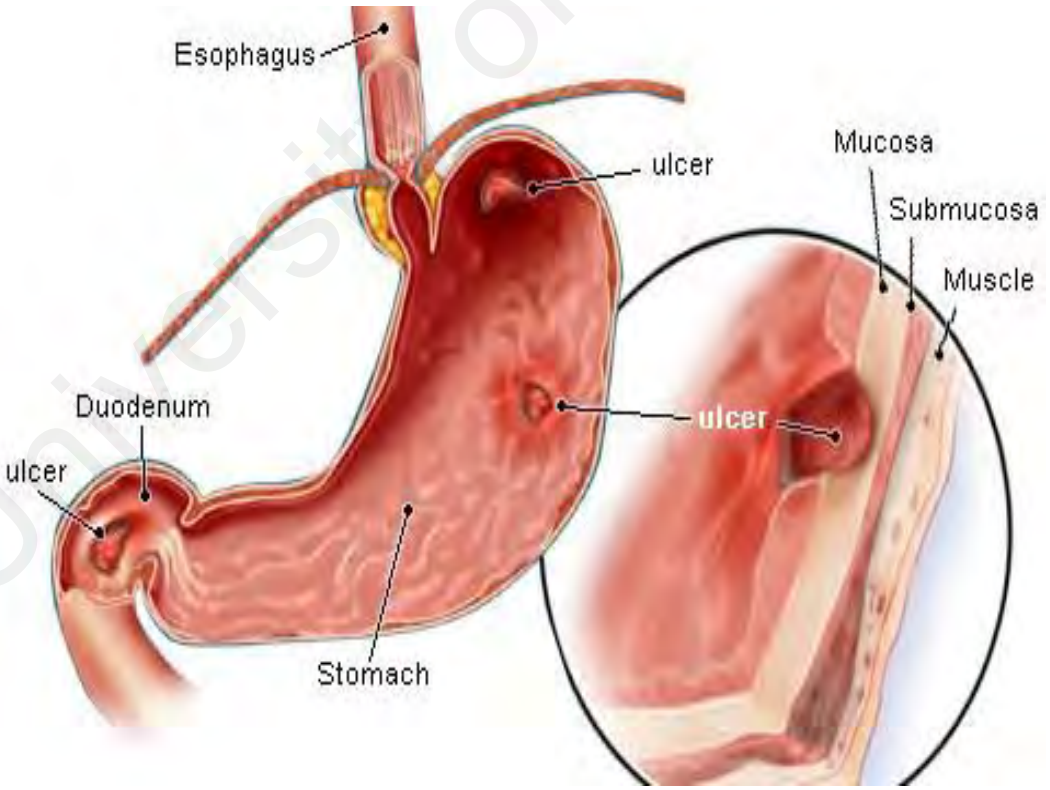

Figure 2.5: Gastric ulcer disease. Adapted from: (Marks, 2012).

2.9. Risk factors gastric ulcer

Under normal situation, the stomach can protect itself from these harmful agents through the defense mechanisms in the gastric mucosa. However, the protection from ulcer can be overwhelmed by aggressive factors that cause mucosal injury. Among aggressive factors as following:

2.9.1. Nonsteroidal anti-inflammatory drugs (NSAIDs)

Non-steroid anti-inflammatory drugs (NSAIDs) are first line therapeutic compounds in the gastric and duodenal ulcers (Bytzer & Teglbjærg, 2001). However, prolonged use of NSAIDs are known to cause adverse effects, particularly reducing natural resistance of the gastric mucosa to ulcerative damage resulting in abdominal pain, diarrhea, stomach upset, and exacerbation of the ulcer (Chan & Leung, 2002; Laine et al., 2008). Use of NSAIDs is known to inhibit the production of prostaglandin resulting in the loss of its protection, leading to a marked increase in the number of gastric and duodenal ulcers. In fact, about 60-94% of long term NSAIDs users, out of which 15%-31% were found to have an established gastric ulcer, with mucosal damage (Wang et al., 2004). The mechanistic implication of NSAIDs in delayed ulcer healing is reported to be due to its effect on the reduced expression of the vascular endothelial growth factor (VEGF) that is said to play an important role in the ulcer healing process (Sato et al., 2010). It is now considered that *H. pylori* infection and NSAIDs usage are among the major etiological causes of peptic ulcer (Lim et al., 2014).

2.9.2. *Helicobacter pylori* infection

The bacterium *H. pylori* infection is one of the most important etiologic agent in the development of gastrointestinal ulcers (Chan & Leung, 2002). *H. pylori* is rod-shaped bacterium that can activate gastrointestinal enzymes and down-regulate mucus and bicarbonate production and release (Nanjundaiah & Annaiah, 2009; Nilsson et al., 2003). This bacterium is capable of changing the gastric environment by reducing acidity to allow for their survival. *H. pylori* can survive in the gastric mucosa for extended periods by penetrating the stomach lining where they are protected from the host immune system by gastric mucus (Ottemann & Lowenthal, 2002). The colonization of the stomach by *H. pylori* results in chronic gastritis. *H. pylori* also produce protease, vacuolating cytotoxin A, and phospholipases that can cause damage to the gastric epithelial cells also resulting in ulcers (Smoot, 1997).

2.9.3. Genetics

The familial predisposition of gastrointestinal ulcers is modest, accounting for approximately 39% of all cases (Räihä et al., 1998). Between 20 to 50% of patients with duodenal ulcers have disease-positive family history, it seems that people genetically prone to acquiring peptic ulcers are not predisposed to *H. pylori* infection (Malaty et al., 2000). However, the incidence of *H. pylori* infections are higher in people with type O blood. A rare genetic relation was shown to occur familial hyperactive pepsinogenemia types I (genetic phenotype causing improved secretion of pepsin) gastric ulcers. This is suggested to be related to gastric acid hyper secretion that causes ulcer development (Atherton, 2006).

2.9.4. Lifestyle

The lifestyle of modern society is fraught with stress and habits detrimental to health like alcohol consumption and cigarette smoking. These social and psychological factors contributes to between 30 to 60% of peptic ulcer cases. Stress-induced peptic ulcer can manifest as hematemesis and melana. In the stomach, the lesions are predominantly multiple hemorrhagic erosions, accounting two-third of all lesions in stress-induced ulcers (Bernd et al., 1978).

Alcohol consumption and cigarette smoking are two factors that are closely related with peptic ulcers (Ko & Cho, 2000). These health threatening habits cause reduced mucus secretion, increased leukotriene B4 level, and increased activity of inducible nitric oxide synthetase, xanthine oxidase and myeloperoxidase that are all contributors to ulcer development. Alcohol, one of contributors to gastric ulcer in humans, causes gastric mucosal irritation, nonspecific gastritis, and gastric reflux (Chisholm, 1998).

2.10. Reactive oxygen species

The body system is influenced by the balance between free radical generation and scavenging. However, the level of free radicals is controlled by enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and coenzyme Q (Brambilla et al., 2008). In the stomach accumulation of free radicals can cause gastric ulcers (Suzuki et al., 2012). In fact, free radicals have been reported to be the main causative factor for gastrointestinal dysfunctions (Bagchi et al., 1998). These radicals attack proteins, DNA and fatty acids on cell membranes (Halliwell & Gutteridge, 1999).

It was also shown that alcohol consumption increases gastrointestinal hydroperoxide and superoxide anion productions, decreasing gastrointestinal mucus and bicarbonate

resulting in cell membrane damage and development of necrotic gastrointestinal lesions. The injury caused by alcohol on gastric mucosa is multifaceted and complicated. Alcohol consumption increases xanthine oxidase and malondialdehyde (MDA), and decrease glutathione content in the gastric mucosa (Marotta et al., 2000) and cause breaks in DNA strands through the production of free radicals (Navasumrit et al., 2000). Alcohol is also associated with imbalance between gastric mucosal protection and action of offensive agents, mucosal edema, cellular exfoliation, sub-epithelial hemorrhages, cell infiltration, inflammation and ulcers (Suzuki et al., 2012), all seems to be mediated through superoxide anion, lipid peroxidation and hydroxyl radical activities in the gastric mucosa (Bagchi et al., 1998).

Gastric mucosal inflammation is a typical effect of alcohol consumption. The inflammatory process induced by alchohol is characterized by excessive release of inflammatory cytokines especially tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) that result in increased production of ROS (Antonisamy et al., 2015). TNF-α is a major pro-inflammatory cytokine secreted by macrophages during gastric ulceration (Martin and Wallace, 2006) and this cytokine stimulates neutrophil infiltration into gastric mucosa during the injury (Wei et al., 2003). IL-6 is another important pro-inflammatory cytokine with pleiotropic nature and plays a central role in acute inflammation and immune regulation (Kishimoto, 2005) by activating neutrophils, lymphocytes and monocytes/macrophage and trigging oxidative stress response while, IL-10 suppresses inflammatory response and inhibits TNF-α production (Mei et al., 2012; Sabat et al., 2010).

2.11. Gastro protective factors

Food in the stomach is digested to chime, move to the duodenal tract for nutrient absorption while unabsorbed materials are excreted as waste in feces. Food may contain constituents that are harmful to gastrointestinal tract and the body. The stomach is protected against the deleterious effects of these constituents by epithelial lining and gastric secretions. The gastric epithelial layer acts as the first line defense against aggressive factors (Wallace, 2008) while the gastric secretion containing mucus, bicarbonate, sulfhydryl compounds, prostaglandins, inflammatory mediators, and epidermal growth factor (Abdel-Salam et al., 2001) protects the stomach mucosa from toxins, poisons, extreme pH and osmolality (Wallace & Granger, 1996).

Mucus on the gastric epithelium is an effective barrier to shave pressure. The carbohydrates of the mucin molecules react with bacteria preventing their colonization while facilitating their aggregation and clearance (Adrian & Flemström, 2005; Singh & Triadafilopoulos, 2005). The bacteria trapped in mucus is excreted in feces. Mucus also has antioxidant activities that protect the mucosa from damage by ROS produced by bacteria and immunocytes (Grisham et al., 1987). There are other roles of mucus to include maintenance of pH on the mucosal surface at near neutral and acting as physical barrier against luminal pepsin (Allen et al., 1993; Allen & Flemström, 2005). The constituents of mucus on the epithelial tissue and luminal tissue also create a moist environment that minimizes attachment of pathogens and absorption of materials (Moncada et al., 2003).

Eicosanoids, the bioactive lipids that include prostaglandins, leukotrienes and thromboxanes play an essential role in gastric physiology because they inhibit gastric acid secretion (Callison et al., 1976; Dey et al., 2006). Previous studies showed that prostaglandin E2 (PGE2) inhibits gastric acid output in mice, rats, dogs, monkeys, and

humans (Main & Whittle, 1973; Newman et al., 1975; Robert et al., 1967; Wallace & Granger, 1996; Wilson, 1990). Prostaglandins also have significant roles in the preservation of mucosal blood flow (Wallace & Mcknight, 1990) and stimulation of bicarbonate and mucus secretion in the stomach (Allen & Garner, 1980). In humans, luminal PGE2 production increased seven-fold over basal values after exposing the stomach to HCl at pH 0.8 (Aly et al., 1985). This effect reiterates the role of prostaglandins in the protection of gastric mucosa to acid damage. In fact, prostaglandins preserve gastric mucosal functions through the stimulation of mucus and bicarbonate excretions, control of acid secretion, stimulation of mast cell infiltration, inhibition of leukocyte infiltration, induction of apoptosis, and protection against ischemia (Atay et al., 2000). It was also shown that pretreatment with prostaglandin reduced injuries and necrotic lesions in the gastric mucosa caused by alcohol (Tarnawski et al., 1985; Trier et al., 1987; Robert et al., 1992).

Other factors that function in the preservation of gastric mucosal integrity include epidermal growth factor, transforming growth factor-beta (TGF-β), and fibroblast growth factor. These peptides also stimulate epithelial cell proliferation in the response to injury and normal tissue turnover (Barnard & Mchugh, 2006). The TGF-β, especially, induces differentiation, proliferation, migration, survival, and angiogenesis. In the human gastrointestinal tract, TGF-β regulates epithelial cell proliferation, inflammation and tissue repair. This was shown by increase in its expression of TGF-β after acute epithelial injury and inflammatory bowel disease (Frey et al., 2006; Hellmich & Evers, 2006).

One of the mechanisms in gastric ulceration is induction of gastric mucosal apoptosis. Thus, one of the means in the prevention of gastric ulceration is the inhibition of premature mucosal cell apoptosis. HSP-70 protein from the heat shock protein family

is ubiquitously present in mammalian cells and this protein serves to defend cells from oxidative stress damage. In cells, HSP-70 preserves structural proteins while functioning in the repair and removal of damaged proteins. In the gastric mucosa, reactive oxygen species (ROS) generation impedes expression of HSP70 making the mucosal layer susceptible to ulcerative damage (Emily et al., 2001).

2.12. Treatment of gastric ulcer

Hospitalization, bed rest, and special diet are prescribed for the treatment of severe gastric ulcers. Development of the disease can be minimized by avoidance of known ulcergenic agents, antacid intake, treatment with proton pump inhibitors, and quitting smoking. There are several other drugs that are used for treating gastric ulcer that functions by neutralizing gastric acid secretion and scavenging free radical. Antacids such as maalox and mylanta are the first optional drugs used to mitigate heartburns and dyspepsia. Unfortunately, antacids are not effective in preventing or curing gastric ulcers. The antacids serve to neutralize gastric acidity through the action of their three basic compounds, calcium, magnesium, and aluminum. The antacids also protect the mucosal layer of stomach by increasing bicarbonate and mucus secretions (Chisholm, 1998). Antibiotics are another group of drugs that can be used to treat gastric ulcers caused by bacterial infection *H. pylori.* An antibiotic such as amoxicillin is effective in decreasing bacterial growth in the stomach (Hentschel et al., 1993).

H2 blockers, including cimetidine and ranitidine, were earlier drugs for treatment of gastric ulcers before the development of proton pump inhibitors (PPIs). H2 blockers are suppressors of gastric acid production by blocking histamine, a cytokine that promotes acid secretion in the stomach. Although H2 blockers are popularly used for gastritis (Chisholm, 1998), these drugs do not heal ulcers (Wallace, 2005). Proton pump inhibitors are fast become the drug of choice for the inhibition of acid production in

gastric ulcer patients (Lin, 2010). The mechanism of action of PPIs is through blocking of stomach acid secretion by suppressing H+/ K+ ATPase, the enzyme responsible for regulating acid secretions by the parietal cells of stomach. Commercially available PPIs are omeprazole (Prilosec OTC), esomeprazole (nexium), lansoprazole (prevacid) and rabeprazole (aciphex) (Wallace, 2005).

2.13. Ethnobotanical Studies on Gastric Ulcer

Medical acceptance of the effectiveness of different plant species could constitute the foundation for the use of these plants as alternative therapies or as main treatment options in situations where no conventional therapy is available (Hutt & Houghton, 1998). The increasing use of medicinal plants is not only because of poverty but also due to the side effects and drug resistance encountered with pharmaceutical drugs. In addition some herbal medicine is safe due to their natural origin, which encourages people to adopt herbs for self-medication (Rokutan et al., 2000). Plants have an extensive capability to amalgamate a huge range of organic compounds, some of which may provide cost effective and better defense against ulcers and entail a lower possibility of recurrence (Koehn & Carter, 2005). Carbenoxolone derived from *Glycyrrhiza glabra* is the first powerful compound confirmed to act against gastric ulcer (Pinder et al., 1976). This finding has triggered further studies focused on discovering other prospective compounds.

Similarly, gefarnate, a substance extracted from cabbage is an antiulcer agent used in traditional medicine (Pillai et al., 2010). Following this, numerous plant extracts have been examined for their advantageous roles in treating gastric ulcers. Noordin and colleagues (2014) reported that *Enicosanthellum pulchrum* extract which is used as traditional medicine for gastrointestinal disorders in Malaysia, exhibit anti-gastric ulcer activity against ethanol induced ulcer. Acyclic polyisoprenoid like geranyl geranyl

acetone (GGA) work effectively against acute gastric mucosal injuries by increasing the production and secretion of gastric mucus (Rokutan et al., 2000). *Zingiber officinale* (ginger) is generally used as a spice, and is acknowledged to have antiulcer and antioxidant attributes in conventional medicine. Many phytoconstituents of ginger, such as sesquiphellandrene (Kobayashi et al., 2001), zingiberene (Ohta et al., 2005), and ar-curcumene (Mózsik et al., 1999), show gastroprotection activity against HCl-ethanol-induced ulcer. Despite numerous studies conducted on the gastroprotective effects of these compounds against gastric ulcers, more studies need to be carried out in order to explore the potentials of natural folkloric compounds like *Clausena excavata*. Thus, investigating the gastroprotective potentials of natural product like *C. excavata* is paramount in order to document its therapeutic effects.

2.14. *Clausena excavata*

Clausena is a genus of about 14 species of evergreen trees, occurring wild mostly in India and tropical Asia (Shier, 1983) including the Himalayas, China, and Peninsula Malaysia (Manosroi *et al*., 2004). In Malaysia *C. excavate* is known as "Cherek hitam" and "Kemantu hitam" (Descola, 1996). In Thailand the herb plant is known as "San Soak." (Khare, 2007). The plant is easy to grow, free of pests and diseases and can withstand heavy pruning (Swarbrick, 1997).

Clausena excavata is a plant species that belongs to the kingdom: Plantae, Phylum: Eudicota, Class: Angiosoermae, Order: Sapindales, Family: Rutaceae, Genus: Clausena, Species: *Clausena excavata.*

Clausena excavata is a slender tree of 10 m in height. The plant is woody with a shiny green leaf (Figure 2.6). The leaves are pinnate; 60 cm long, with 10-15 pairs of dark green narrowly oval oblique leaflets, 3.5 to 7 cm long and pointed tips. The leaves

have a characteristic curry-like smell when crushed. *C. excavata* has striking hourglass-shaped gynophores that is completely glabrous. The flower is consists of four membranes with short pedicel, 4 mm in diameter. The fruit is approximately 19 mm in length and the twigs finely hairy (Swarbrick, 1997).

2.14.1. Traditional Usages of *Clausena excavata*

Amongst Malays, *C. excavata* is of medicinal importance (Ali et al., 2000). The plant is traditionally used in the treatment of abdominal pains, snakebites, and as a detoxification agent. The leaves of this plant are used in the treatment of malaria, stomachache, cold, headache, pulmonary tuberculosis, wound, dysentery, and diarrhea (Zheng et al., 2013). The pounded root is used as a poultice for sores. The root and stem decoction are drunk for bowel complaints, mainly colic. Decayed teeth can be treated using dried and powdered rootstock of the plant. In Indonesia, expressed juice of *C. excavata* is used as vermifuge for coughs (Arbab et al., 2011). In Malaysia, the plant is used for the treatment of yaws (Gimlette & Thomson, 1939) while the flowers and leaves are boiled and the decoction consumed by mothers after childbirth (Grieve and Scora, 1980).

Figure 2.6: *Clausena excavata* Burm. f. (Rutaceae); fruits and leaves; source: http://www.tradewindsfruit.com

2.14.2. Phytochemical and Pharmacological Properties of *Clausena excavata*

The pharmacological properties of compounds extracted from *C. exacavata* are being extensively investigated (Blumenthal *et al*., 2000). *C. excavata* was reported to have compounds with some of the highest beneficial biological activities among the *Clausena* genus (Table 2.2). The plant also contains other pharmacologically active compounds like coumarin, carbazole alkaloid, and glycosides. The phenolic and flavonoid compounds from the plant possess immunomodulatory, analgesic, anti-inflammatory, antiviral, anticancer, antioxidant, antimycobacterial, anti-malarial, and antifungal activities. (Rahman et al., 2002; Sunthitikawinsakul et al., 2003; Manosroi et al., 2004; Sharif et al., 2011; Guntupalli et al., 2012; Kumar et al., 2012; Arbab et al.,

2012). Based on these biological properties of chemical consitutents of *C. excavata,* it is postulated that the *C. excavata* leaves extract is effective in the healing of wounds and gastric ulcers.

Table 2.2: Biologically active compounds of *Clausena excavata*

Compoud name	Chemical class	Bioactivity	Reference
Clausine-B	carbazole alkaloid	Anticancer	Wan et al., 2005
Clausine-TY	carbazole alkaloid	Anticancer	Taufiq-Yap et al, 2007
Clausenidin, Nordentatin,	Pyranocoumarins	Anticancer, antibactrialial	Ali et al., 2000
Xanthoxyletin, Murrayanine	Carbazole derivatives	Antibactrialial	Sunthitikawinsakul et al., 2003
Mukonal	Limonoid	Antifungal	Takemura et al., 2000
Dentatin	Coumarin	Anti-HIV-1	Ali et al., 2000
Clausene-D	Alkaloid	Antiplatelet	Wu & Furukawa, 1982
Sansoakamine	Carbazole alkaloid	Anti-malarial	Lastra et al., 2005

CHAPTER 3: MATERIALS AND METHODS

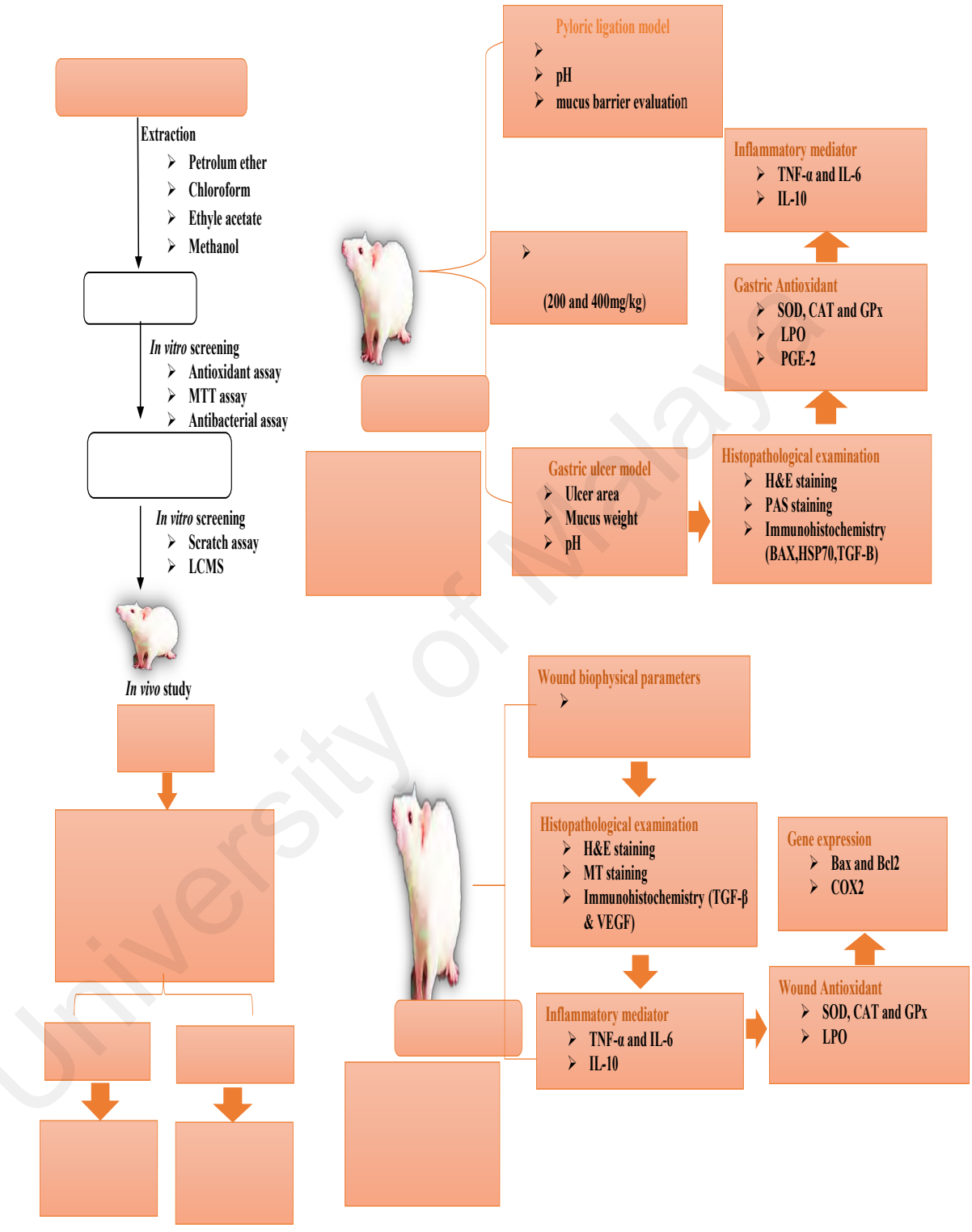

Figure 3.1: Study desigen

3.1. Collection and Processing of Plant Material

3.1.1. Plant Collection and Identification

Clausena excavata leaves were collected from Pendang, Kedah, Malaysia. The plant was identified and authenticated by Dr. Shamsul Khamis of the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia. A voucher specimen (TI-013201-CE) was deposit at Institute of Bioscience (IBS), Universiti Putra Malaysia.

3.1.2. Extraction of Plant Material

The leaves were sorted, washed and dried at room temperature for two weeks before ground into powder and stored in air tight plastic bags. Extraction was done at room temperature, in the order of petroleum ether (PT), chloroform (CH), ethyl acetate (EA), and methanol (MOH). The extraction at 1: 5 (weight-to-volume) of dried plant weight to petroleum ether was allowed to stand for 3 days to ensure the total penetration of the solvent and dissolved chemicals. The mixtures were then filtered using a filter paper (Whitman, 185 mm). The filtrate and residue were subject to further extraction with chloroform, ethyl acetate, and methanol and the filtrate collected after each extraction. All filtrates were evaporated to dry under reduced pressure using rotary evaporator (Buchi, Switzerland) at 45 to 50°C to obtain crude extracts. The yield of dried extracts was calculated and stored at – 20^{o}C until use.

3.2. In vitro screening of extracts for Antioxidant Activity

3.2.1. Ferric Reducing Antioxidant Power Method (FRAP)

Ferric Reducing Antioxidant Power (FRAP) reagent containing 25 mL of acetate buffer (pH 3.6), 2.5 mL of 10 mM 2, 4, 6-Tripyridyl-s-Triazine (TPTZ) solution and 2.5 mL of 20mM $FeCl_3 \cdot 6H_2O$ was freshly prepared. One milligram of extract was dissolved in 1 mL of absolute deoxymethylsulfoxide (DMSO) (Fisher Scientific, UK). Ten microliter of $FeSO_4$ solution, standard, or fractions were placed in the designated microplate wells in triplicates. 300 µL of reagent was added to each well and the optical density of reaction mixture determined spectrophotometrically at 593 nm after 5 min. The FRAP value was determined from the $FeSO_4 \cdot 7H_2O$ standard curve (APPENDIX C) and expressed as µM ferrous/mg sample (Benzie & Strain, 1996).

3.2.2. 2, 2-Diphenyl-1-picryllyhydrazyl (DPPH) Free Radical Scavenging Method

The DPPH scavenging activity of *C. excavata* leaves fractions were estimated using the method described by Generalic *et al*., (2011). Briefly, 195 µL DPPH reagent was added to 5 µL of each fractions or quercetin standard. The mixtures were incubated in the dark for 2 h at room temperature. The optical density of the mixture determined at 515 nm against a blank. The DPPH scavenging activity calculated using the following formula:

$$\text{DPPH scavenging (\%)} = \frac{\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{blank}}} \times 100.$$

Where $\text{Abs}_{\text{blank}}$ = Absorbance of blank; $\text{Abs}_{\text{sample}}$ = Absorbance of sample.

The IC_{50} (inhibitory concentration of sample required to scavenge 50% of the DPPH free radicals) values for each sample was obtain by linear regression analysis of the dose response curve.

3.2.3. Total Phenol Content

The total phenol content (TPC) of each fraction was determined as described by (Mayakrishnan et al., 2013) using Folin-Ciocalteu reagent. Ten microliter of 1mg/mL fraction was added to 100 µL of 10% Folin-Ciocalteu reagent in water, mixed well and left to incubate for 5 min at room temperature. One hundred microliter of 10% sodium carbonate was added and the mixture incubated for 2 h at room temperature. The optical density was measured spectrophotometrically at 750 nm and results were obtained from the Gallic acid as a standard expressed as mg Gallic acid equivalent (GAE)/g extract (APPENDIX C).

3.2.4. Total Flavonoid Content

Total flavonoid content (TFC) was estimated using the method described by (Adedapo *et al.* 2008). Five hundred microliter of each fractions was added to 500 µL of 2% AlCl3 dissolved in ethanol. After 1 h incubation at room temperature, the optical density was determined at 420 nm. The TFC was determined from the quercetin standard curve and expressed as quercetin equivalent/g extract (APPENDIX C).

3.3. Evaluation of cytotoxicity using (3-(4, 5- dimethylthiazol-2-yl)-2, 5 Diphenyl tetrazolium bromide assay

The diphenyl tetrazolium bromide (MTT) assay was used to determine cell toxicity of *C. excavata* extracts (Riss *et al.,* 2004). The target cells selected were human immortal keratinocyte HaCaT, macrophage (J77A.1), and Vero cells (3T3), obtained from American Type Culture collection, USA, was cultured in RPMI-1640 or DMEM

growth medium supplemented with 4500g glucose/L, 110mg sodium pyruvate/L and 1% penicillin– streptomycin (Sigma-Aldrich, UK) and 10% fetal bovine serum (FBS) (BIOWEST, France). The cells were cultured at 37°C in under 5% CO_2 (NuAire, Plymouth, MN, USA). The cells were subcultured until they reached 80% confluence and seeded in a 96-well plate at a density of 6.0×10^3 cells/well. After 24 h of incubation, the cells were washed with phosphate buffered saline (PBS). The extract was diluted in the growth media of the particular cell line. The vehicle for initial stock of drug was 0.1% DMSO. The extracts at concentrations of 12.5, 25, 50, 100, 200, and 400 µg/mL were added to their designated cells and incubated at 37°C under 5% CO_2 (NuAire, USA) for 24 h. Twenty microliters of MTT reagent was added to each well and the plate re-incubated for 4 h. The purple formazan formed was solubilized with 150 µL DMSO in the dark at room temperature for approximately 30 min to dissolve the yellow MTT tetrazolium salt to the purple formazan salt. The number of viable cells was determined by measuring the optical density spectrometrically determined at 570 nm, with reference at 630 nm, in a microplate reader (Tecan, Austria). Each experiment was repeated three times with triplicate wells for each concentration.

3.4. Assessment of Antibacterial Activity

Crude extracts of *C. excavata* were evaluated for antibacterial activity against gram positive and gram negative bacteria.

3.4.1. Disc diffusion method

The disc diffusion method was carried out using the technique of Kirby–Bauer (Bauer *et al*., 1966). Gram-positive bacteria, *Staphylococcus epidermidis, Bacillus subtilis* and Gram-negative bacteria, *Acinetobacter anitratus* and *Pseudomonas aroginosa* were obtained from the Institute of Bioscience, Universiti Putra Malaysia.

Bacterial suspension was prepared from overnight culture, two colonies were selected with a sterile loop and transferred in to 2ml of normal saline and vortex thoroughly, and then turbidity is compared to that of the 0.5 McFarland standard solution (containing about 1.5×10^8 CFU/mL). The bacteria suspension was spread uniformly on the surface of the nutrient agar using a sterile cotton swabs. Extracts at 1mg in 10 uL aliquots was dispensed into each 6 mm sterile discs and seeded on the surface of the inoculated plate using sterile forceps. The plates were incubated at 37°C for 24 h. The antibacterial activity was measured by the development of inhibition zone around the disc. The diameter of this clear zone was measured in millimeters (mm). Streptomycin and DMSO were used as positive and negative control, respectively.

3.4.2. Determination of minimum inhibitory concentration (MIC)

This was conducted according to method of Agoramoorthy et al. (2007). Serial dilution between 0.009 mg /mL to 20 mg fractions per milliliter was prepared then placed in microplate wells. A 24h culture diluted with a sterile 0.9% normal saline and 0.5mL McFarland standard was used to achieve inoculums of 10^6 CFU/ mL. The samples were inoculated with 10µl standard suspensions of tested bacteria homogenized and incubated for 24 hours, then 5 µl was withdrawn from each well, sub cultured on nutrient agar plates and incubated at 37 °C for 24 h. Following incubation in broth microdilution method, 30 µL of 0.01% resazurin was added into each well of the 96-well microtiter plates and the plates were subjected to incubation for another two hours.

Color change of the dye from blue to pink indicated cell viability. The MIC was determined as the lowest concentration of fractions in the broth medium that did not show any growth of the test bacteria, which changed the resazurin colour from blue to pink or purple, whereas the minimum bacterocidal concentration (MBC) was recorded as the minimum concentration of the extract that did not change the blue pigmentation of resazurin as well as kills bacteria after the period of incubation.

3.5. Scratch Wound assay of Methanolic fraction *C. excavata*

This experiment was carried out according to method described by Guler *et al*., (2014). Fibroblast cells (3T3) ATCC layered in a 24-well plate at 3×10^5 cells/mL and grown in a 10% FBS media until at least 80% cell confluent was reached. Scratches on the fibroblast layer were made with a 200 µL a sterile pipette tip**.** Cells were then rinsed with PBS to remove the loose cell debris. One hundred microgram per milliliter of MOH extract was added to each well and the plate incubated. The scratch in the wells was then examined at 0, 24 and 48 h post-treatment under a phase-contrast microscope (Leica microsystems, Germany) and digital images captured using a digital camera (Leica, Microsystems, UK). The width of the scratch was measured at each time period to determine rate of cell migration. The scratch test assay was performed in triplicates.

3.6. Phytochemical Characterization of the Methanolic *C. excavata* Leaves Extract

The methanolic fraction showed good antioxidant activity and phenolic content, optimal proliferation and migration of cell lines, and antibacterial activity was subsequently selected for phytochemical screening.

Ultra-high resolution liquid chromatography–tandem mass spectrometry (UHPLC) system was used in the identification and characterization of active constituents in the methanolic fraction *C. excavate*. The analytical condition consisted of a C18 column (150 mm x 4.6 mm X, particle size 5µM), mobile phase made up from Water (solvent A) and Acetonitrile (solvent B) [0.1% formic acid and 5 mM ammonium format for each], a flow rate of 0.8 mL/min, and an injection volume was set to 20 µL. The gradient program was adjusted to perform a serial dilution from 10 to 90% B from 0.01min to 8 min and back to 10% B in 0.1min after holding the system for 2 min, while maintaining the system for 5 min for re-equilibrated. The run-time was 15 min. The diode array detector recorded the spectra range from 100 to 1200 nm to identify molecular ion. All chromatic peaks from MS analyzer were identified by matching with ACD Labs mass spectral library and corresponding literature data (Mayakrishnan *et al.*, 2013). A data dependent program was established in the liquid chromatography-tandem mass spectrometry analysis such that the most abundant ions in each scan were selected and subjected to MS/MS analysis.

3.7. *In vivo* Acute Toxicity Study of Methanolic fraction of *C. excavata*

The acute oral toxicity of the methanolic fraction was evaluated according to the criteria of the Organization for Economic Cooperation and Development (OECD-423) guidelines (OECD, 2001). This test is used to describe the adverse effects of a substance that may result from single exposures within 14 days.

3.7.1. Experimental Animals

Thirty-six adult male and female Sprague-Dawley rats, 8 to 10 weeks old, weighing between 165 and 200 g, were procured from the Animal House of the Faculty of Medicine, University of Malaya, Malaysia. The rats were maintained in clean stainless steel cages at an ambient temperature of 25±2°C and fed standard rat/mouse pellet (Specialty Feeds, Glen Forrest, and Western Australia) and water *ad libitum* for one week before experimentation. All studies performed were approved by the Institutional Animal Care and Use Committee, University of Malaya (ISB/22/007/2013/1111/SFA).

3.7.2. Animal Grouping

The Animals were divided into six groups; control male (CM) (n=6), control female (CF) (n=6) given 10% Tween-20, male 1 (M1) (n=6) and Female 1 (F1) (n=6), given 2000 mg/kg body weight of methanolic fraction *Clausena excavata* (MFCE), male 2 (M2) (n=6) and female 2 (F2) (n=6), given 5000 mg/kg body weight MFCE. The MFCE was suspended in 10% Tween-20 and given orally to each rat. The rats were observed for abnormal behavior, such as sedation, convulsion, diarrhea, and respiratory distress, during the first 3 h post-treatment. There was no mortality and the rats humanely euthanized after 14 days with an overdose of CO_2.

3.7.3. Blood Parameters

Before euthanasia, the rats were fasted for at least over night and blood samples were collected in plain and EDTA tubes. Serum was separated by centrifugation 3000 rpm (Hettich, EBA 20) for 10 min and used for determination of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, bilirubin, creatinine (Cr), urea (BUN), sodium (Na^+), potassium (K^+), and chloride (Cl^-). Anticoagulated whole blood samples were used for determination of erythrocyte (RBC) and leukocyte (WBC) counts and hemoglobin concentrations.

3.7.4. Histopathology

Liver and kidney tissue samples were collected from all the experimental groups, weighed, and then fixed in 10% buffered formalin for 48 h, embedded, sectioned (Leitz Watzlar), stained with hematoxylin and eosin stain, and observed under light microscopy (Appendix A).

3.8. Wound healing of Methanolic fraction *C. excavata*

3.8.1. Experimental Animals

Thirty adult male Sprague-Dawley rats (200–250 g) were used in the study. The rats were acclimatized for 2 weeks in a well-ventilated room, and given standard pellet feed and water *ad libitum* throughout the experimentation period.

3.8.2. Animal Grouping and Wound infliction

The rats were divided into five groups of sex animals each, and anesthetized with an intramuscular injection of the 100 mg/mL ketamine and 30 mg/mL xylazine combination prior to skin wounds infliction. Skin from the dorsal nape of the neck was shaved with an electrical clipper, disinfected with 70% alcohol, and injected with 0.1 mL 2% lignocaine hydrochloride. A 2 cm diameter circular area (~3.14 cm^2) of the skin was made using a circular stamp, and full thickness of the marked skin was then cut carefully, to ensure the muscles are spared (Figure 3.2), using a pair of sterile scissors and forceps.

The wounds were left exposed and undressed. Group 1 (negative control group) was treated with vehicle (gum acacia in normal saline 20 mg/mL); Group 2 was treated with 0.2 mL intrasite gel (Smith and Nephew Ltd., UK) (positive control); Groups 3, 4, and 5 were treated with 0.2 mL MFCE at concentrations of 50 (MFCE-LD), 100 (MFCE-

MD), and 200 (MFCE-HD) mg/mL. All treatments were by topical application twice daily for 14 days beginning on first day of wound infliction.

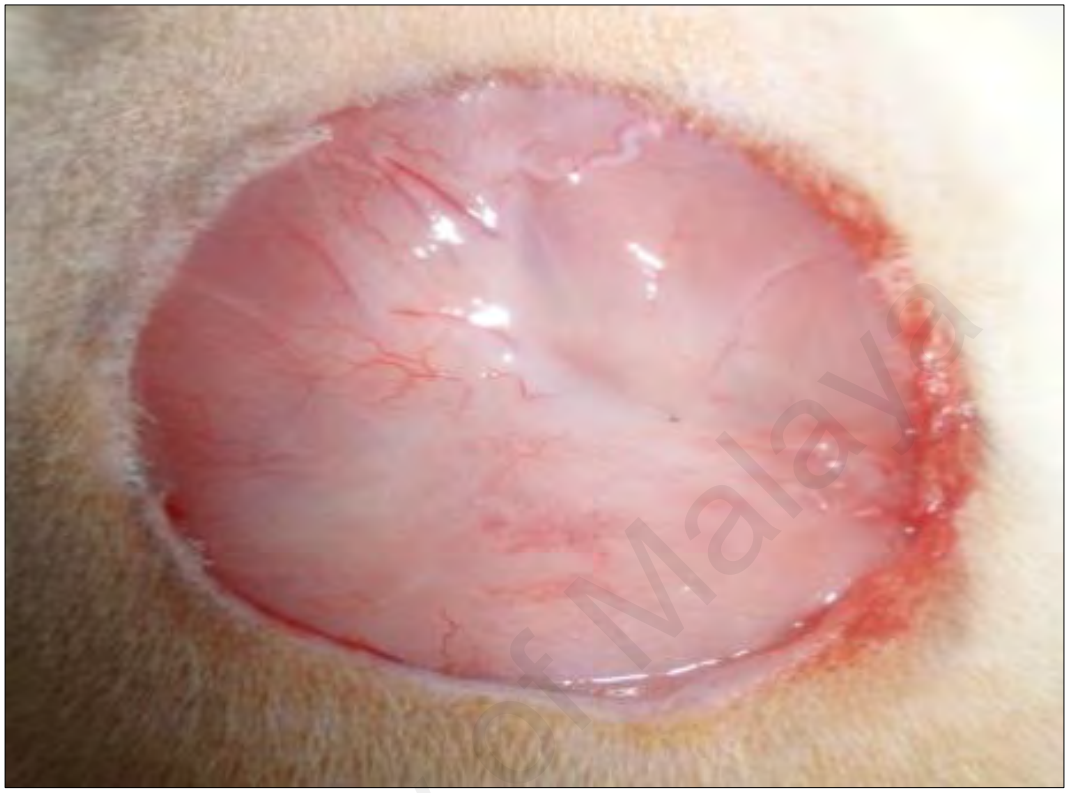

Figure 3.2: Inflicted skin wound at the nape of the dorsal neck of rats.

3.8.3. Wound contraction Assessment

The wound area was measured every 5 days for 15 days by placing transparent paper over the wound and tracing its border. The tracing was superimposed on a 1 mm^2 graph paper to determine area according to the method described by Ikobi *et al*. (2012). The area of the contracting wound with treatment is expressed as a percentage of the initial wound size according to the following formula:

$$\text{Wound contraction (\%)} = \frac{\text{Area}_{\text{intial}} - \text{Area}_{\text{treatment}}\,(\text{mm}^2)}{\text{Area}_{\text{initial}}} \times 100$$

Where $\text{Area}_{\text{initial}}$ = Initial wound area, $\text{Area}_{\text{treatment}}$ = Wound area with treatment.

Differences in wound healing of the treated groups are derived by comparing with healed wound area of the control group, during the treatment period (Murthy *et al*., 2013).

3.8.4. Histopathological examination

3.8.4.1. Hematoxylin and Eosin- and ’ trichome-stain (MT)

After euthanasia, skin specimens were collected from all the rats and placed in 10% buffered formalin, processed in the automated tissue processing machine (Leica, Germany), and 5 μm square tissues sectioned using a microtome (Leica, Germany). The tissues were stained with hematoxylin and eosin (H&E) and Masson’s trichrome (MT) (Appendix A). For each group, 6 microscopic fields were examined each from three slides at 40× magnification. The H&E-stained sections were examined for epithelialization (epithelial proliferation in the epidermal layer), degeneration (epidermal cells showing vacuolation or vacuolar degeneration), granulation tissue

(necrotic and inflammatory cells), and leucocytic (polymorphonuclear and mononuclear) infiltration and images captured. The lesions, based each of the above, were scored according to five-point scale: 0 – normal, 0%; 1 – occasional evidence, 25%; 2 – light scattering, 30%–50%; 3 – abundant evidence, 55%–75%; and 4 – confluent cell, 80%–100% (Atiba *et al*., 2011).

The MT sections were similarly evaluated according to color intensity; 0 - no coloration, 1 – low, 2 - dull, 3 - moderate, and 4 – bright. Fibroblast distribution and collagen maturity were also determined as follows; 0 - no collagen, 1 - semi-thin fibers, 2 - thin fibers, 3 - semi-thick fiber, 4 - thick fibers. Evaluation of angiogenesis was based on the distribution of new blood capillaries and collagen distribution was based on the distribution of collagen fibers, using the five-point scale described by (Atiba *et al*., 2011).

3.8.4.2. Immunohistochemistry Staining for VEGF and TGF-β

Immunohistochemical staining was conducted according to the manufacturer's protocol (Dako Cytomation, USA). Tissue sections were prepared as described earlier for histopathology. The tissue sections were deparaffinized and immersed in 100, 80, 70, and 50% alcohol in order for 5 min each before hydrating in PBS twice for 5 min each time. The sections were boiled in 10 mM Tris buffer (pH 9.0) for 15 minutes to retrieve the antigen, the slides cooled, and incubated for 10 mins with 3% H_2O_2. The slides were then washed twice in PBS and incubated with rabbit polyclonal primary antibodies to VEGF or TGF-β1, (Abcam, UK) at a dilution of 1:500 and 1:100, respectively, and the tissue was then incubated in a humidified chamber at 4 °C for 1 h for detection of VEGF and 18 h for TGF-β1. Horseradish peroxidase-conjugated secondary antibodies (EnVision System) were added to the sections and the slides were incubated at room temperature for 60 minutes.

The tissues were then stained with 3, 3′-diaminobenzidine tetrahydrochloride (DAB) and substrate (Dako, CA, USA) for 5 min. All washings were done with 0.1% Tween-20 in PBS, except for washing after rehydration and after chromogen incubation (DAB), in which PBS without Tween-20 was used. The slides were counterstained with hematoxylin, and subsequently dehydrated in increasing concentrations of alcohol of 50, 70, 80, and 100% before clearing in xylene. Tissue sections were air-dried and mounted onto slides for examination under light microscopy. Dark-brown stained VEGF-positive cells in six fields/tissue sections from 3 rat/group were counted at 40× magnification. The extracellular matrix (ECM) distribution of TGF-β1 was evaluated using a four-point scale: 0 – 0% ECM; 1 – 25 – 40% ECM; 50 – 65% ECM; 70 – 100%, according to method described previously (Atiba *et al*., 2011).

3.8.5. Antioxidant activity and lipids peroxidation

Tissues samples were collected from all the animals at day 15 post-wound infliction for the determination of tissue superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and lipid peroxidase activities. After homogenization, the mixture was centrifuged using a refrigerated centrifuge Rotofix 32 (Hettich Zentrifugen, Germany) at 4000 rpm at 4°C for 20 min. The supernatant was collected for enzymatic antioxidants evaluation. The protein concentration was determined using the Bradford method with bovine serum albumin as a standard (Bradford, 1976) (Appendix C).

3.8.5.1. Superoxide dismutase activity

The tissues were homogenized (Omni TH, USA) in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (1 mM EGTA, 210 mM mannitol, and 70 mM sucrose/g tissue), pH 7.2. The homogenate was then centrifuged at 1,500 × *g* for 5 min at 4°C, and the supernatant collected and assayed for SOD activity using the Cayman assay kit (Cayman Chemical, USA). Appendix B.

3.8.5.2. Catalase activity

Tissue samples were homogenized (Omni TH, Omni) in cold potassium phosphate buffer, pH 7.0, containing 1 mM EDTA. The mixture was then centrifuged at 10,000× *g* for 15 min at 4°C, and the supernatant assayed for CAT activity using the Cayman assay kit (Cayman Chemical, MI, USA). Appendix B.

3.8.5.3. Glutathione peroxidase activity

Tissue samples were homogenized (Omni TH, Omni) in 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM dithiothreitol. The homogenate was then centrifuged at 10,000× *g* for 15 minutes at 4°C. The supernatant was assayed for GPx activity using the Cayman assay kit (Cayman Chemical, Ann Arbor, MI, USA). Appendix B

3.8.5.4. Lipid peroxidation activity

The lipid peroxidation (LPO) assays (Cayman Chemical, MI, USA) were carried out according to manufacturer's instructions. Tissue samples were homogenized (Omni TH, Omni) in PBS, and extraction buffer provided with the kit to extract lipid hydro peroxides. Real-time chromogenic reaction was allowed to occur for 5 minutes. The absorbance was read at 500 nm. Appendix B

3.9. Evaluation of TNF-α -6 and IL-10 via enzyme –linked immunosorbent assay

To determine TNF-α (Item No.CSB-E11987r), IL-6 (Item No. CSB-E04640r), and (IL-10) (Item No. CSB-E06897Rb) in the homogenate, 100 mg skin tissue was weighed and homogenized on ice in 1 mL cold PBS buffer. The tissue homogenate was centrifuged (Hettich Zentrifugen, Germany) at 10,000 × g and 4°C for 10 min and the supernatant was collected and kept at (–80 °C) until use. Detection of cytokine in the supernatant was by a commercial ELISA (CUSABIO, China) according to manufacturer's instruction. Briefly, 100 µL of sample or standard per well was placed in a 96-well sterile ELISA microplates coated with capture antibody. The plates were sealed and incubated for 2 h at 37°C. The sample was aspirated, and then100 µL biotinylated-antibody (1:100 dilutions) was added at each well and the plates were sealed and incubated for 1 h at 37°C. The medium was aspirated from the wells and the plate washed with 200 µL buffer each time for three times. The plates were then incubated with 100 µL of working solution of horseradish-peroxide conjugated streptavidin (1:100 dilutions) for 1 h at 37°C. After aspiration of the supernatant, the washing step was repeated and 100 µL of the substrate solution tetramethylbenzidine (TMB) was added to each well and the plate incubated for 30 min at 37°C in the dark. The reactions were stopped with 50 µL of stop solution. The optical density (OD) was determined in the ELISA plate reader (Universal Microplate reader, USA) at 450 nm.

3.9. Quantitative Real-time polymerase chain reaction (RT-PCR) BAX, BCL2 and COX-2 gene (wound healing model)

The HD-MFCE healed rat skin wound was collected and placed in RNA later solution (Ambion, USA) and snap-frozen in liquid nitrogen, and kept at -80°C.

3.9.1. RNA extraction

Total RNA was extracted from skin samples and purified using RNeasy fibrous tissue mini kit (Qiagen, Cat. No: 74704). The extraction and purification processes were carried out as per kit protocol. Briefly, 60 mg of skin tissue sample was weighed and placed in 2 mL microfuge tube, homogenized using a tissue ruptor in a volume 600 µL RLT Buffer (containing 1% B-mercaptoethanol), followed by addition of 10 µL diluted proteinase K. The homogenate was vortexed before incubating at 55°C for 10 min on a hot plate (Benchmark, USA) and centrifuged for 3 min at 10000 × g. The supernatant was transferred to a 1.5 mL RNase free micro-centrifuge tube. Then 0.5 volume (250 µL) of 96 - 100% ethanol was added to the supernatant, mixed and the 700 µL of the solution transferred to the mini RNeasy purification column in a 2-mL collection tube, and finally centrifuged at 8000 × g for 15 sec. The spin column was transferred to a new collection tube and 350 µL RW1 buffer added and mixture centrifuged at 8000 × g for 15 sec. To avoid genomic DNA contamination, the mixture was treated with 80µL DNaseI (Qiagen, Germany). Then 350µL of RW1 buffer was added and the mixture centrifuged at 10000 × g for 15 sec. The purification spin column was placed into a new collection tube and 500 µL of RPE buffer1 was added and the mixure centrifuged at 10000 × g for 2 min. Finally, the spin column was placed in the 1.5 ml RNAase free collection tube to elute the RNA by adding 20 µL nuclease-free water and centrifuging at 10000 × g for 1min. The RNA collected was kept at -80°C until use. The RNA concentration determination by optical density at 260nm. The quality of RNA extracted

(A260/A280 ≥ 1.65) were measured using a Nano Drop ND- 2000 spectrophotometer (Thermo Fisher scientific). The integrity of RNA was determined using agarose gel electrophoresis (Bio-Rad, USA) and viewed after staining with ethidium bromide (Pfaffl *et al*., 2003).

3.9.2. Reverse transcription (RT)

Reverse transcription was carried out using a commercial kit (Tetro cDNA synthesis kit, applied Bioline, Cat.No65042). In each 20 µL reaction mixture, the total RNA sample was mixed with RT reaction mix (Table 3.1) and the reaction determined in the thermal cycler (MJ Research PTC-100 Thermal cycler, USA) at 25°C for 10 min followed by 45°C for 30 min. The reaction was stopped at 85°C for 5 min and was held at 4°C. The cDNA product was then stored at -20°C until use.

3.9.3. Real-time amplification assay for gene expression

The reaction setup for all gene expression assays was performed according to the manufacturer's instructions for the Universal SYBR Green PCR Kit (BIO RAD Cat. No: 1725270) and generated by the CFX Manager System Software (Ver. 1.6, BioRad Laboratories). The reaction was carried out on ice by mixing 12.5 µL 1X universal SYBR Green super mix with 1 µL 10X corresponding primers and 1 µL cDNA and the reaction volume made to 25 µL with nuclease-free water (Table 3.2). The reaction mixture was gently mixed by pipetting and the PCR reaction run using the RT-PCR machine (MJ Research PTC-100 Thermal cycler, USA) (Table 3.3).

Table 3.1: Composition of the reverse transcriptase reaction	
Reaction mixture	Volume (μL)
RNA sample	Up to 9
Random Hexamer	1
dNTP mix	1
RT Buffer	4
RNase inhibitor	1
Tetro Reverse Transcriptase	1
Nuclease-free water	To 20

Table 3.2: Genes used in the study

No.	Gene name and abbreviation	NCBI Reference Sequence
1.	BCL2-associated X protein (Bax)	NM_017059.1
2.	B cell lymphoma -2 (BCL2)	NM_016993.1
3.	Cyclooxygenase-2 (Cox-2)	NM_017232.3
4.	Glyceraldehyde-3-phosphate dehydrogenase(GAPDH)	NM_017008.3
5.	Act-β (β-actin)	NM_031144.2

Table 3.3: Thermal cycle condition

parameters	Polymerase activation	PCR (40 cycle)	
	Hold	Denature	Anneal/extend
Temperature(°C)	95	95	60
Time (mm:ss)	05:30	00:15	00:30

The PCR gradient was done for all genes simultaneously in one run to determine the optimal annealing temperature. Annealing temperature gradients were 50 to 60 °C for 30 sec. Standard curve was created for each gene to determine the efficiency of gene amplification. No template control (NTC) contained all the components of the reaction except the cDNA. Samples that give higher CT value than control is considered to be gene down-expression and *vice versa*. CT values were calculated as the mean of triplicate measurements. The comparative CT quantification was by the ΔΔCT method (Shandiz *et al*., 2015). The comparative CT method (ΔΔCT) expression of genes was normalized with the endogenous housekeeping genes (β-actin and GAPDH). The real-time RT-PCR analytical results for a given gene are expressed as the difference in ΔΔCT value between treated and calibrator (untreated control). The fold change was calculated as follows:

$$CT_{target} - CT_{control} = \Delta CT$$

$$\Delta CT_{sample} - \Delta CT_{calibrator} = \Delta\Delta CT$$

$$\text{Fold change} = 2^{-\Delta\Delta CT}$$

Where CT_{target} = CT of test sample; $CT_{control}$ = CT of endogenous control; $CT_{calibrator}$ = CT of untreated control.

3.10. Gastro protective activity evaluation of *C. excavata*

3.10.1. Animals

Fifty-four male Sprague-Dawley rats (220 to 240 g), aged 6 weeks were purchased from the Animal Resource Centre, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The rats were housed in groups of 3/cage and allowed to acclimatize for week, with free access to commercial feed and water. The experiment was conducted under a constant ambient temperature of approximately 22°C and 12 hour light/dark cycle and with strict compliance to the guidelines of the Institutional Animal Care and Use Committee, University of Malaya (ISN/22/007/2013/1111/SFA). All animal studies were performed under sterile conditions at the Experimental Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

3.10.2. Plant preparation and dose selection

The MFCE, omeprazole, ethanol were suspended, at 5ml/kg with 5% Tween 20 v/v, and Tween 20 was used as a vehicle (Nordin, *et al*., 2014). The doses of MFCE used for treating the rats were according to the OECD 423 guidelines and those described by Venkateswalu and Rao (2013). Two doses namely, 200 and 400 mg/kg body weight were chosen from the acute toxicity study.

3.10.3. Pyloric ligation model

The anti-secretory effect of MFCE was determined in rats according to the method described by Shay (1945) with slight modifications. Twenty-four Sprague-Dawley rats were assigned to four equal groups, fasted of feed for overnight but with free access to water. The rats were then treated once via oral gavage as follows: group 1: 5% Tween 20 (v/v) (negative control); group 2: 20 mg/kg body weight, omeprazole dissolved in 5% Tween 20 (v/v); (Positive control); group 3: 200 mg/kg body weight MFCE

dissolved in 5% Tween 20 (v/v); and group 4: 400 mg/kg body weight MFCE dissolved in 5% Tween 20 (v/v).

The rats were anesthetized, via intramuscular route, with a combination of ketamine (50 mg/kg body weight) and xylazine (5mg/kg body weight). The abdomen was opened by making a small midline incision below the sternum. The pyloric portion of the stomach was slightly lifted and ligated, avoiding traction to the pylorus or damage to blood vessels. The stomach was then carefully put back in place and the abdominal wall closed using sutures. After 4 h, all rats were sacrificed with overdose CO_2, their stomachs were immediately removed and the volume and pH of gastric contents, determined. Total acidity of the gastric content was determined by titration with 0.01 N sodium hydroxide (NaOH) solution using phenolphthalein as indicator and calculated using the formula: Total acidity (mEq/L) = Volume of NaOH solution × 0.01 × 36.45 × 1000 (Tan et al., 2002). Although the pH is related to total acidity, pH measures acid strength while total acidity measures the amount of acid in the gastric juice (Zakaria *et al.*, 2012).

The gastric mucus content was determined according to the method described by (Alhaider *et al*., (2007). The glandular part of the stomach was firstly weighed then soaking for 2 h in 10 mL 0.1% w/v alcian blue solution (0.16 M sucrose, 0.05 M sodium acetate at a pH 5.6). The stomach was washed with 10 mL 0.25M sucrose solution twice, for 15 min and then 45 min, remove unbound dye. The dye incorporated into the mucus was extracted by soaking in 10 mL 0.5 M magnesium chloride for 2 hours with periodic shaking at 30 min intervals. The extract was mixed with an equal volume of diethyl ether then shaken vigorously (Labsol, India) for 2 min. The resulting emulsion was centrifuged at 3000 rpm (Hettich Zentrifugen, German) for 10 min. The alcian blue

in the bottom layer was read at 580 nm and the result obtained from the standard curve (Appendix B) was expressed as μg/g of stomach tissue.

3.10.4. Rat gastric ulcer model

Thirty male Sprague-Dawley rats (220 to 240 g), were randomly divided into 5 groups of 6 rats each and maintained in wire bottomed cages, fasted for over night, and deprived of water for 2hr. The rats were orally pretreated as follows; Group 1: vehicle [5% Tween 20% (v/v), 5 mL/kg body weight] (normal control); Group 2: 5 mL/kg body weight absolute ethanol (ulcer control); Group 3: 20 mg/kg body weight omeprazole in 5% Tween 20 (v/v) (positive control); Group 4: 200 mg/kg body weight MFCE in 5% Tween 20 (v/v); Group 5: 400 mg/kg body weight MFCE in 5% Tween 20 (v/v). One hour after pre-treatment, all rats, except the normal controls, were orally gavaged with 5 mL/kg body weight absolute ethanol (Nordin *et al*., 2014), anaesthetized via intramuscular route with a combination of ketamine (50 mg/kg) and xylazine (5mg/kg), euthanized under overdose CO_2 and their stomachs immediately removed (Sidahmed *et al*., 2015.

3.10.5. Gastric mucosa gross lesion

Gastric ulcer appears as elongated bands of hemorrhagic lesions on the stomach mucosal surface. The ulcer area was determined under a dissecting microscope with a square-grid eye piece at 1.8× magnification. The area of each ulcer lesion was measured by counting the 2 × 2 mm squares. The lesion total area for each stomach was calculated from the following formula: Ulcer area (mm^2) = (sum of small squares counted) × 4 × 1.8. The inhibition percentage was then calculated as described by Al-Amin (2012) using the following formula:

$$\text{Ulcer inhibition (\%)} = \frac{UA_{control} - UA_{treated}}{UA_{control}} \times 100\%$$

Where UAcontrol = gastric ulcer area of untreated control rats; $UA_{treated}$ = gastric ulcer area of treated rats.

3.10.6. Gastric mucus content and juice acidity

The gastric mucosa was gently scrapped using a glass slide to obtain mucus and gastric mucosal tissue weighed using an electronic balance. Gastric juice was collected and centrifuged at 4,000 × g at room temperature for 10 min. The acidity of the supernatant was determined using a digital pH meter (Indran *et al*., 2008).

5.10.7. Histopathology and lesion evaluation

3.10.7.1. H&E- and periodic acid-Schiff (PAS)-stain

A small piece of gastric mucosa tissue with ulcer was fixed with 10% buffered formalin solution and dehydrated with alcohol and finally embedded in paraffin wax. Five micrometer tissue sections were cut and stained with haematoxylin and eosin (H & E) for light microscopic examination (Appendix A). Lesions such as hemorrhage, submucosal edema, epithelial erosion, and inflammatory cell infiltration were determined according to the previously described scoring system (Laine &Weinstein, 1988; Khaleel *et al*., 2014) using the following categories: 0 = normal, 1 = Mild (less than 1/3 of field involved), 2 = Moderate (between 1/3 and 2/3 of field involved), and 3 = severe (more than 2/3 of the field involved).

Tissue sections were also stained with periodic acid-Schiff (PAS) stain (Luna, 1968) to determine mucus production (Appendix A). Tissue with mucus stained magenta and six microscopic focal fields at × 20 magnification were captured from each slide and used

for the image J software (http://imagej.nih.gov/ij/). The results were expressed as percentage of area stained magenta in color to total mucosal area (Sidahmed et al., 2015).

3.10.7.2. Immunohistochemistry for BAX, Heat shock protein 70, and TGF-β

Tissue sections were deparaffinized for 15 min at 58°C before immersing in decreasing concentrations of 100, 80, 70 and 50% alcohol for 5 min at each concentration, followed by hydration in phosphate buffered saline (PBS) twice for 5 min each time. The sections were then heated in 10 mM tris buffer (pH 9.0) for 15 min to retrieve the antigens. The slides were allowed to cool to room temperature and endogenous peroxidase was blocked by incubating the sections with 3% H_2O_2 for 10 min at room temperature. The slides were then washed twice with PBS and incubated with rabbit primary anti-HSP70, anti-BAX or TGF-β antibody (Abcam, UK) at dilutions of 1:500, 1:200 and 1:800, respectively. The slides after incubation in a humidified chamber, in a dark, at room temperature for 30 min, were immersed in 0.1% Tween-20 in PBS (PBST) twice for 5 min each time. Horseradish peroxidase-conjugated secondary antibodies (EnVision+ System) were then added to the sections, and the slides incubated at room temperature for 60 min. The sections were then incubated with the substrate, 3,3'-diaminobenzidine tetrahydrochloride (Dako, USA), allowed to stand at room temperature for 5 min, counter-stained with Harris hematoxylin, dehydrated with 50, 70, 80 and 100% alcohol, and finally cleared in xylene. Air-dried sections were mounted for light microscopic examination.

Six microscopic focal fields at × 20 magnification were captured from each slide and used for the image J analysis using the image J software (http://imagej.nih.gov/ij/). Five selected area from each tissue were analysed (Sidahmed et al., 2015). Each replicate

from the same group were evaluated and mean± S. E. M of the stained areas (μm2) determined (Collins, 2007).

3.10.8. Measurement of proteins content of stomach tissue

To prepare the stomach tissue homogenate, 0.2 g of whole gastric tissue was weighed and homogenized on ice with 2 mL of PBS using tissue ruptor (Qiagen, Germany) for 15 min. After homogenization, the mixture was centrifuged at 2000 × g in a refrigerated centrifuge at 4°C for 20 min. The protein content of tissues was estimated using Bradford assay (Bradford, 1976).

3.10.8.1. Prostaglandin E2 (PGE2)

The gastric prostaglandin was estimated in the homogenate using a prostaglandin E_2 kit (Cayman Chemical, USA). This test is based on competitive binding between PGE_2 and PGE_2-acetylcholinesterase (AChE) conjugate (PGE_2 Tracer) for PGE_2 monoclonal antibody. Briefly, the assay was performed by mixing 50 μL standard solution or sample homogenate, with 50 μL AChE tracer and 50μL expressing monoclonal antibody. The plate was covered with a plastic film and then incubated at room temperature for 1 h in the orbital shaker (Wisd, Germany) followed by one hour incubation with 200μL Ellman's reagent reconstituted with 20 mL ultra-pure water. The plate was read at 412 nm in a spectrophotometer (Shimadzu-Japan) and the results expressed as PGE_2/mg of protein.

3.10.8.2. Antioxidant Activity

The levels of SOD, CAT, GPx and LPO in the stomach tissue homogenate were estimated as previously described in sections **3.8.5.1, 3.8.5.2, 3.8.5.3** and **3.8.5.4**, respectively.

3.10.8.3. TNF-α -6, and IL-10

The concentrations of TNF-α, IL-6, and IL-10 in the stomach homogenate was analysed as described in section **3.9.**

3.11. Statistical analysis

Data obtained from the experiments were expressed as mean ± SE, SD (standard error or standard division of mean), and subjected to one-way analysis of variance using SPSS (Version 19.0; IBM Corporation, Armonk, NY, USA) and Graph Pad software (Version 5.04; San Diego California USA) with significance at $P<0.05$. The analyzed data were then presented in tables and bar graphs as shown.

CHAPTER 4: RESULTS

4.1. Yield of *C. excavata* Crude extracts

Extraction of air-dried ground *C. excavata* leaves with petroleum ether, chloroform, ethyl acetate, and methanol produced an extract yield (wt/100gm) of 1.56, 2.57, 0.38, and 0.94%, respectively.

4.2. *In vitro* antioxidant activity of *C. excavata* extracts

The antioxidant activity of *C. excavata* petroleum ether, chloroform, ethyl acetate, and methanol extracts were evaluated by ferric reducing antioxidant power (FRAP) and 2, 2-diphenyl-1-picrylhydrazyl Free Radical Scavenging (DPPH) tests. The level of total phenolic and total flavonoid contents were also determined.

4.2.1. Ferric Reducing Antioxidant Power

The study showed that methanolic and ethyl acetate extracts at 3 mg/mL possessed greater (p<0.05) antioxidant potential among all fractions (Figure 4.1). The FRAP value of *C. excavata* methanol fraction is 4649 ± 11.3 μmol Fe^{2+}/mg, while that for ethyl acetate, chloroform, petroleum ether and quercetin were 4501 ± 69.32, 2307.85 ± 61.33, 955.85 ±6.3 and 7008± 42.1 μmol Fe^{2+}/mg, respectively.

4.2.2. 2, 2-Diphenyl-1-picrylhydrazyl Free Radical Scavenging

The radical scavenging activity was assessed using a DPPH free radical test and are presented in Figure 4.2. The study showed that the MOH extract possessed the highest radical scavenging activity among extracts at 86.89 ± 1.4 % compared to ethyl acetate, chloroform, petroleum ether and quercetin at 61.79 ± 0.61, 33.03 ± 0.3, 78.74 ± 0.66, and 80.1 ± 0.66 % respectively. The MOH has the lowest IC_{50} at 23 µg/mL extract compared to ethyl acetate, chloroform, petroleum ether extracts at 64, 83, and 397µg/mL for ethyl acetate, and chloroform, and petroleum ether *C. excavata* extracts, respectively. The IC_{50} for quercetin was 28µg/mL.

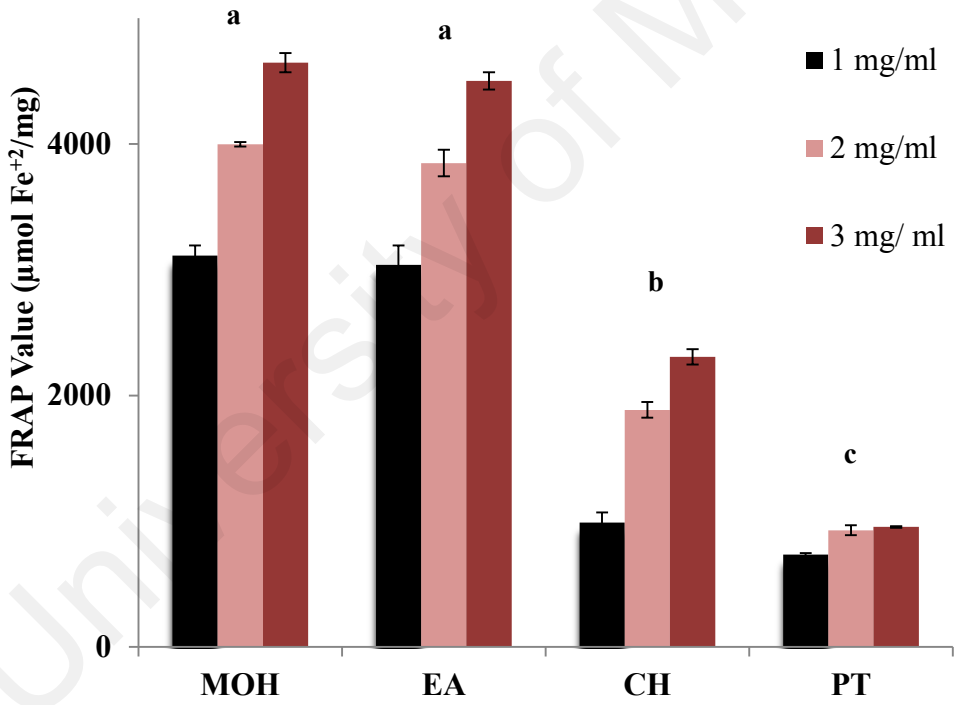

Figure 4.1: Ferric-reducing antioxidant power (FRAP) of *C. excavata* leaves solvent extracts. MOH = methanol; EA = ethyl acetate; CH =chloroform; PT = petroleum ether. [a,b,c,d] Mean ± SD with different superscripts are significantly different at $P< 0.05$.

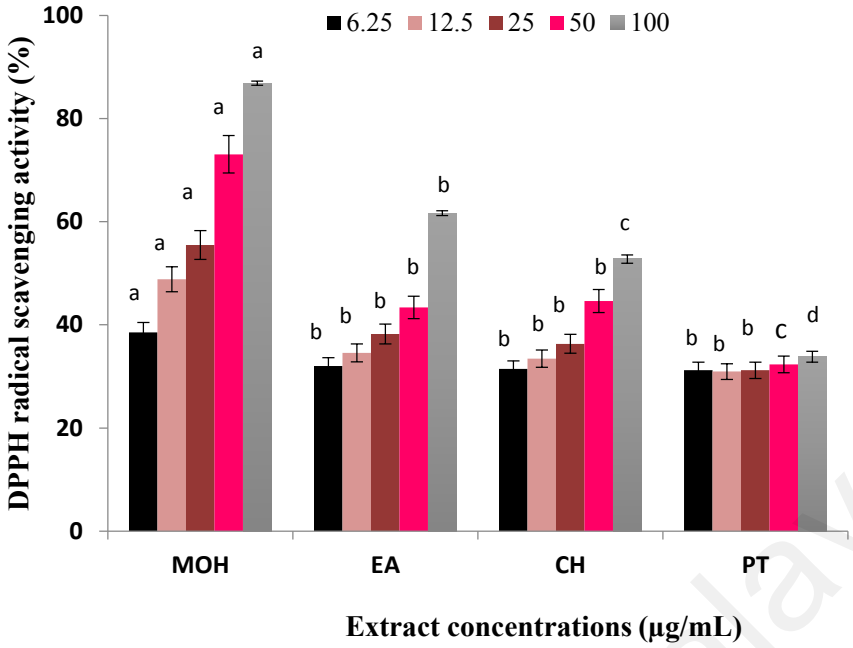

Figure 4.2: 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of *C. excavata* leaves extracts. MOH = methanol; EA = ethyl acetate; CH = chloroform; PT = petroleum ether. [a,b,c,d] Mean ± SD with different superscripts are significantly different at $p < 0.05$.

4.2.3. Total Phenolic and Flavonoid Contents of *Clausena excavata*

The MOH fraction had the highest amount of phenolic compounds among extracts, while the chloroform extract had the highest total flavonoid content (Table 4.1). The results revealed the total phenolic content of MOH as 522.0 ± 11.6 mg GAE/g and the total flavonoids content at 96.7 ± 2.0 mg QE/g, while total phenolic content was 497.0 ± 7.1, 373.0 ± 6.9 and 157.0 ± 7.5 mg GAE/g and total flavonoid content was 142.5 ± 1.0, 188.6 ± 3.0 and 25.8 ± 0.8 mg QE/g for ethyl acetate, chloroform and petroleum fractions, respectively.

Table 4.1: Total phenolic and flavonoid contents of *C. excavata* fractions.

	Solvents			
	Methanol	**Ethyl acetate**	**Chloroform**	**Petroleum ether**
TPC	$522.0^{a} \pm 11.6$	$497.0^{b} \pm 7.1$	$373.0^{c} \pm 6.9$	$157.0^{d} \pm 7.5$
TFC Q	$96.7^{c} \pm 2.0$	$142.5^{b} \pm 1.0$	$188.6^{a} \pm 3.0$	$25.8^{d} \pm 0.8$

Values are mean ± SD. ^{a,b,c,d} Mean ± SD with different superscripts are significantly different at $p < 0.05$. TPC = total phenolic content; TFC = total flavonoid content; GAE = Gallic acid equivalent; QE = quercetin equivalent.

4.3. *In vitro* Cytotoxicity of solvent extracts

We investigated the effect the *C. excavta* leaves extracts on the cytotoxicity of the HaCaT (keratinocyte), Vero (fibroblast) and J77A.1 macrophage cells (Figure 4.3). The ethyl acetate and chloroform extracts were more toxic to all cell lines. The ethyl acetate began showing cytotoxicity at 100 µg/mL while the chloroform extracts at 200 µg/mL.

The MOH and EA extracts of *C. excavata* were slightly toxic to Vero cells at 400µg/mL only, while CH and PT *C. excavata* extracts were innocuous to these cells at the same dose (Figure 4.3B). However, proliferation of the HaCaT and Vero cells increased following exposure to the extracts at low doses. The cytotoxic effect of MOH on J774A.1 cells is shown in (Figure 4.3C). At 200 and 400 µg/mL, MOH was not toxic to J774A.1 cells. The result suggests that MOH is safe to be used for in vivo rat model. Thus, this study was paramount in order to study the cytotoxic effect of the four fractions on these cell lines to choose the best extract in the investigation of the expression of cytokines, acute toxicity, wound healing properties, gastro protective effect and LCMS profiling to identify the active constituents.

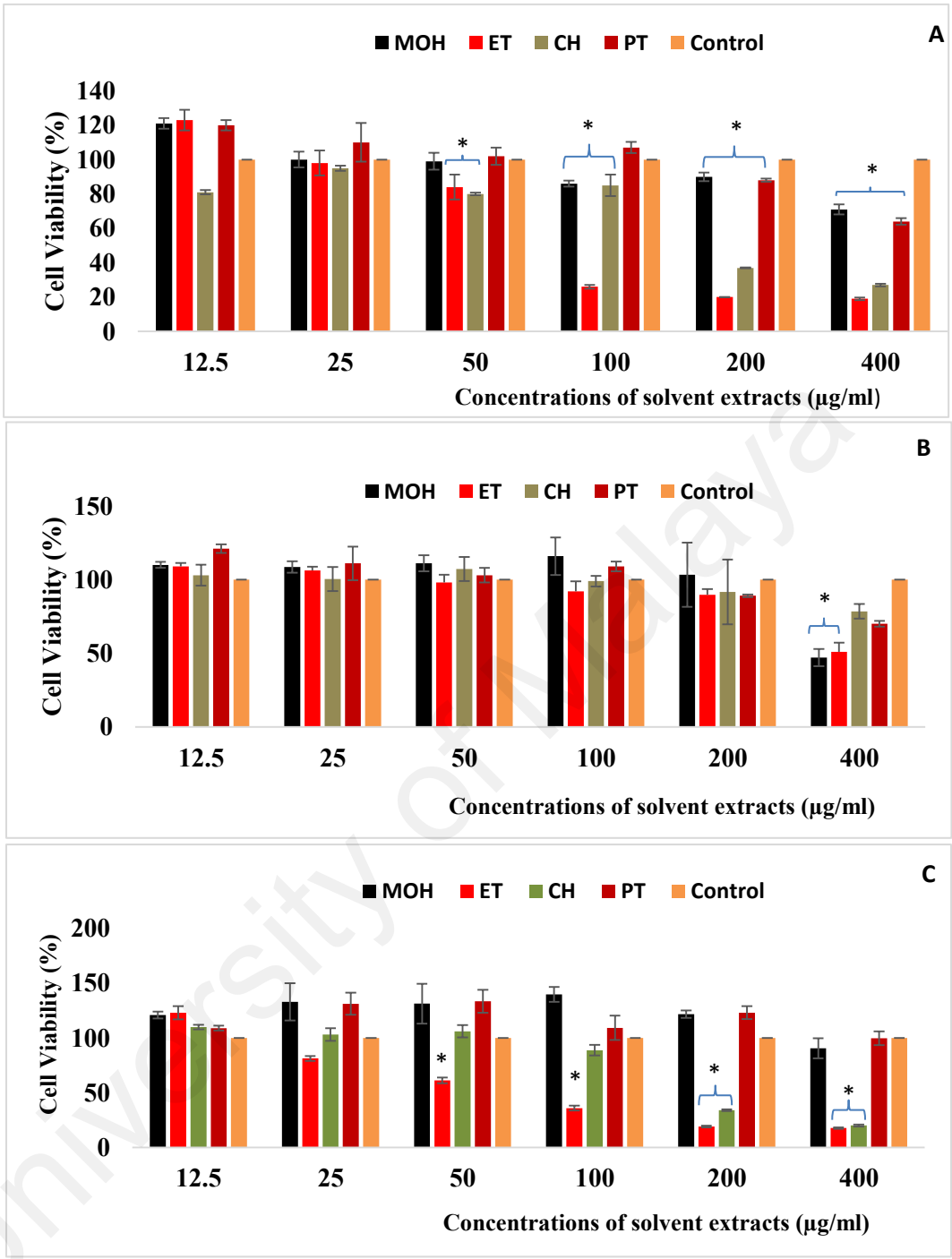

Figure 4.3: Cell viability evaluation following MTT assay on **(A)** HaCaT, **(B)** Vero cells and **(C)** J77A.1 cells different *C. excavata* leaves extracts. Ethyl acetate and chloroform *C. excavata* extract were most cytotoxic to HaCaT and J77A.1 cells.*significantly different versus control group, $P \leq 0.05$.

4.4. Antibacterial activity of extracts

The results of the assay showed in Table 4.2 that *B. subtilis* showed relative resistance to MOH extract only and were susceptible to chloroform, and ethyl acetate *C. excavate* extracts. *S. epidermidis* was susceptible to all extracts. *P. aeruginosa* was highly resistant to all extracts among the Gram-negative bacteria.

Table 4.2: Inhibition zone for *C. excavata* fractions.

***C. excavata* extract**	**zones of inhibition (mm)**			
	GM+ve		**GM-ve**	
	S. epidermidis	***B. subtilis***	***A. anitratus***	***P. aeruginosa***
Petroleum ether	-	-	-	-
Chloroform	15.7 ± 0.7	12.1 ± 0.2[b]	14.1 ± 0.33	-
Ethyl acetate	15.9 ± 0.71	11.5 ± 0.2[b]	13.7 ± 0.50	-
Methanol	13.4 ± 0.2	9.7 ± 0.1[a]	13.3 ± 0.16	-
Streptomycin(+ve control)	34.0 ± 0.3	26.5 ± 0.2	27.2 ± 0.09	28.3 ± 0.5
10%DMSO(-ve control)	-	-	-	-

- no inhibition.

The minimum inhibitory concentration (MIC) is considered as the lowest concentration of a compound that inhibited visible growth of bacteria. Subsequently, methanol, ethyl acetate and Chloroform were selected for the determination of minimum inhibitory concentration (MIC). The MIC value of plant extracts of *C. excavata* against the test bacteria ranged from 0.019 mg/mL (ethyl acetate) to 0.078 and 1.25 mg/mL (Chloroform and methanol), respectively. The MIC values of ethyl acetate extract ranged from 0.019 mg/mL to 0.039 mg/mL with the least MIC values compared to other crude extract fraction. This is illustrated in table 4.3.

Table 4.3: Minimum inhibitory concentration of *C. excavata* extract

***C. excavata* extracts**	**Minimum inhibitory concentration (mg/mL)**		
	S. epidermidis	***B. subtilis***	***A. anitratus***
Methanol	0.078	1.25	0.078
Ethyl acetate	0.039	0.039	0.019
Chloroform	0.078	0.078l	0.078

MBC value of methanol and chloroform fractions from leaves extract of *C. excavata* were 0.156 mg/mL against *S. epidermidis, B. subtilis* and *A. anitratus* except methanol fraction was 2.5 mg/mL for *B. subtilis* followed by ethyl acetate extract that ranged from 0.039 mg/mL against *A. anitratus* to 0.078 mg/mL against *S. epidermidis* and *B. subtilis* (Table 4.4).

Table 4.4: Minimum bactericidal concentration of *C. excavata* leaves extract

***C. excavata* extracts**	**Minimum bactericidal concentration (mg/mL)**		
	S. epidermidis	***B. subtilis***	***A. anitratus***
Methanol	0.156	2.5	0.156
Ethyl acetate	0.078	0.078	0.039
Chloroform	0.156	0.156	0.156

4.5. Scratch wound assay of Methanolic Extract of *C. excavata*

Proliferating phase initiated by the proliferation and migration of fibroblast, which plays a critical role by creating new collagen and contracting the wound. Thus, Methanolic fraction was subjected to wound scratch test assay to prove if can contribute to enhancement the wound-healing process by stimulation the migration of fibroblast.

It was observed that the migration of fibroblasts in and around the scratch area was faster and more prominent after 100 µg/mL MOH treatment compared to the untreated cells. The distance the cells proliferated to cover the scratch area was estimated in relation to the 0 h appearance. The scratch was completly closed after 48 h post-MOH treatment while in the untreated scratch, the gap was still obvious by the time (Figures 4.4 a and b), showing that wound closure was significantly (p<0.05) faster after MOH treatment than in the untreated scratch.

4.6. Phytochemical Characterization of the Methanolic Extract of *C. excavata*

From the above *in vitro* antioxidant, cell proliferation, antimicrobial, and wound healing propriety results, MOH showed good antioxidant activity and phenolic content, optimal proliferation and migration of cell lines, therefore it was further evaluated for its phytochemical characterization.

Based on LCMS/MS analysis, the MFCE contains pipecolic acid, myricetin glucoside conjugate, quercetin-rhamnose-hexose-rhamnose, kaempferol conjugate, furocoumarin, and 8-geranyloxy psoralen (Table 4.5).

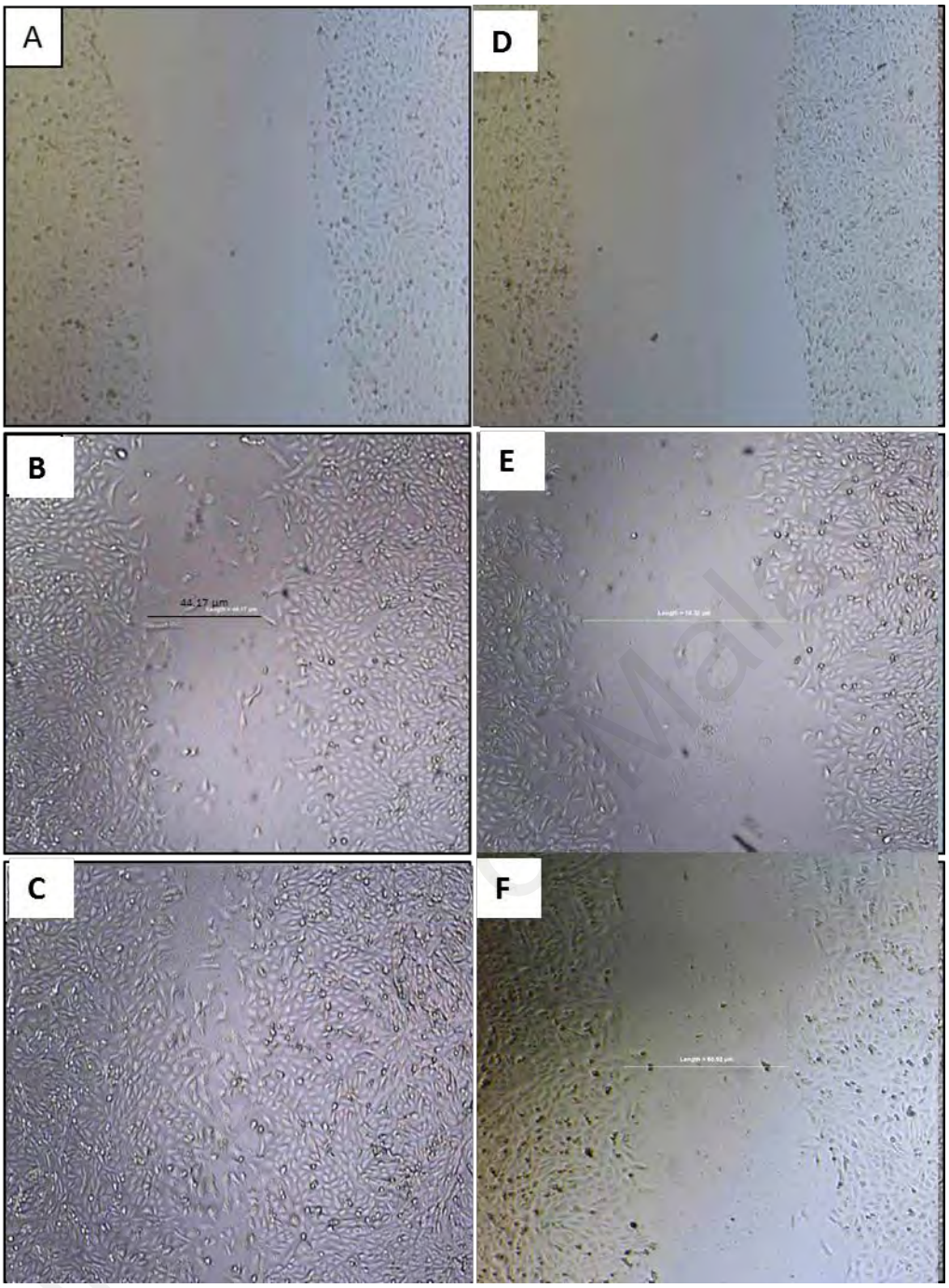

Figure 4.4 (a): *In vitro* wound scratch assay of fibroblasts treated with 100 µg/mL methanolic *C. excavata* extract (MFCE). Fibroblasts treated with MFCE showed faster cell migration than untreated cells. A= Control; B= 24 hrMFCE; C= 48 hr MFCE; D= Control; E= 24hr untreated; F= 48 hruntreated (4x).

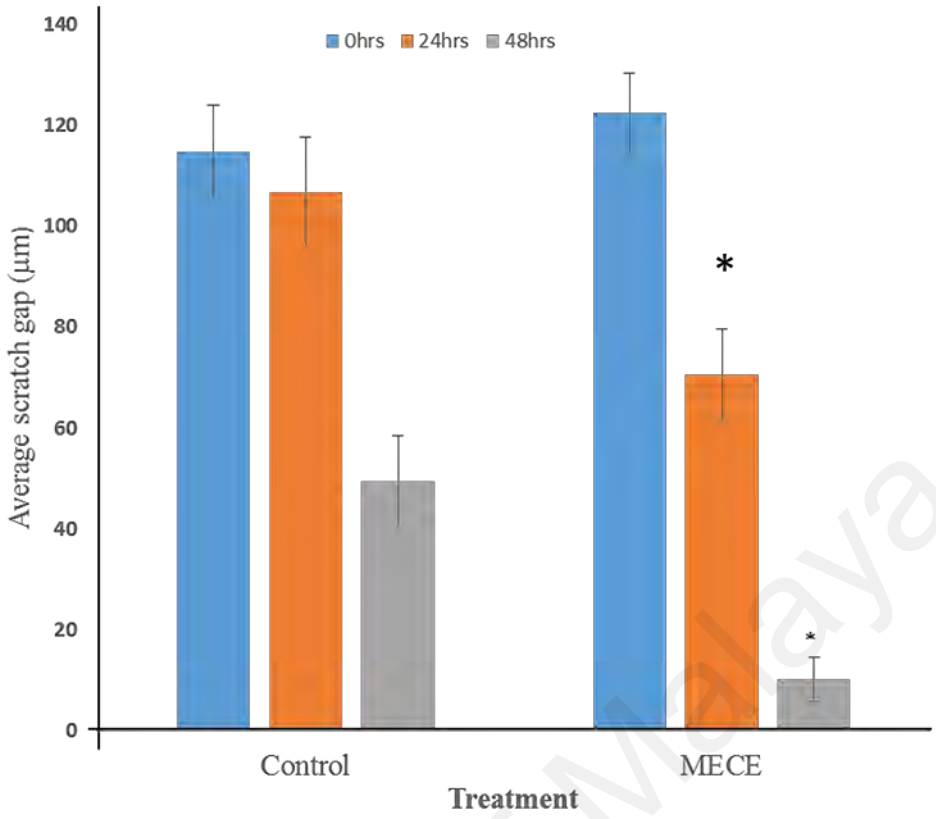

Figure 4.4 (b). Migration of fibroblast cells in *in vitro* wound scratch assay after treatment with 100 µg/mL methanolic fraction *C. excavate* (MFCE). *Significantly different compared with control ($P < 0.05$).

Table 4.5: Compounds identified from MFCE via LCMS/MS analysis

Retention time	Molecular weight	MS/MS fragments ions[M-H]-,[M+H]+	Tentative Identification	References
3.69	756.2	(-)755, 300, 271.0, 255.1, 179.0, 151.0	Quercetin-rhamnose-hexose-rhamnose	Lee et al.,2011 Kachlicki et al.,2008 Daniele et al.,2004
3.9	772.2	(-)771, 316, 271.1, 179.0, 151.0	Myricitin glucosideConjugate Myricetin 3-O - rhamnosyl-glucoside 7- O –rhamnoside	Lee et al., 2011 Kachlicki et al.,2008 Daniele et al., 2004
4.3	740.2	(-) 739.2, 284.1, 255.0, 179, 151.0, 179.0,	Kaempferol conjugate	Lee et al.,2011 Yang et al.,2011
7	179.3	(+) 161.2, 135.1,133.1	Caffeic acid	Lee et al.,2011
6.72		(+) 77.0, 91.0, 95.0, 105.0, 119.1, 134.1, 148.3, 149.2, 156.3, 161.3, 174.4, 177.3, 179.3, 190.4, 202.4, 204.4, 206.4, 218.4, 221.4, 230.4, 232.4, 243.4, 245.4, 246.4, 256.4, 270.4, 284.4, 288.4, 298.4, 316.4, 326.4, 344.4, 362	8-Geranyloxy psoralen	

Table 4.5: Continued

Retention time	**MS/MS fragments ions[M-H]-,[M+H]+**	**Tentative Identification**
5.95	(+) 77.91, 91.0, 95.0, 111.0, 119.0, 133.1,147.2, 149.2, 161.2, 177.2, 179.3, 191.3, 203.3, 243.3, 245.4, 247.3, 255.3, 271.4, 273.4, 289.4, 291.4, 313.4, 323.4, 331.4, 341.4, 359.4, 377.4	Unidentified Furocoumarin
5.27	(+) 117.1, 134.2, 146.2, 147.2, 150.2, 160.3, 163.3, 180.3, 202.3, 246.4, 473.4, 491.5, 509.5, 527.6	Unidentified Flavonoids
4.72	(-) 107.0, 123.0, 137.1, 139.1, 163.1, 179.1, 195.2, 197.2, 241.1	Unidentified Phenolic acid

4.7. Acute Oral Toxicity Study of Methanolic Extract *C. excavata*

During the 14-days experimental period, there was no mortality or behavioral abnormality in the rats treated orally with 2000 and 5000 mg/kg body weight MFCE. Thus the oral LD50 was greater than 5000 mg/kg body weight. The body weight was lower in the rats treated with 5000 mg/kg of the extract. The kidneys and liver of rats treated with the extract did not show any gain in relative of organ weights (Table 4.6), and there was no significant (p>0.05) body weight change (Figure 4.5a and b).

Table 4.6: Relative weights of selected organs after administration of a single dose methanolic *C. excavata* leaf extract.

Organ	Sex	MFCE (mg/kg body weight)		
		Untreated	2000	5000
		Relative organ weight (g)		
Liver	Male	2.33±0.10	2.55±0.19	2.58±0.20
	Female	2.60±0.07	2.78±0.14	2.82±0.16
Kidney	Male	0.68 ±0.01	0.74 ±0.02	0.70 ±0.01
	Female	0.55 ±0.01	0.61±0.02	0.63±0.03

Values are mean ± SD. MFCE = Methanolic extract *C. excavata*. No significant difference among means within or across rows. Untreated = rats given normal diet.

There was slightly increased in serum liver enzyme concentrations in both male and female rats fed MFCE. In male rats treated with 5000 mg/kg body weight MFCE, there were significant elevations in ALP, AST, and ALT concentrations, while in female rats similarly treated, only the ALT and ALP concentration increased significantly (p<0.05). The total serum bilirubin concentration increased in female rats treated with 2000 and 5000 mg/kg body weight MFCE (Table 4.7).

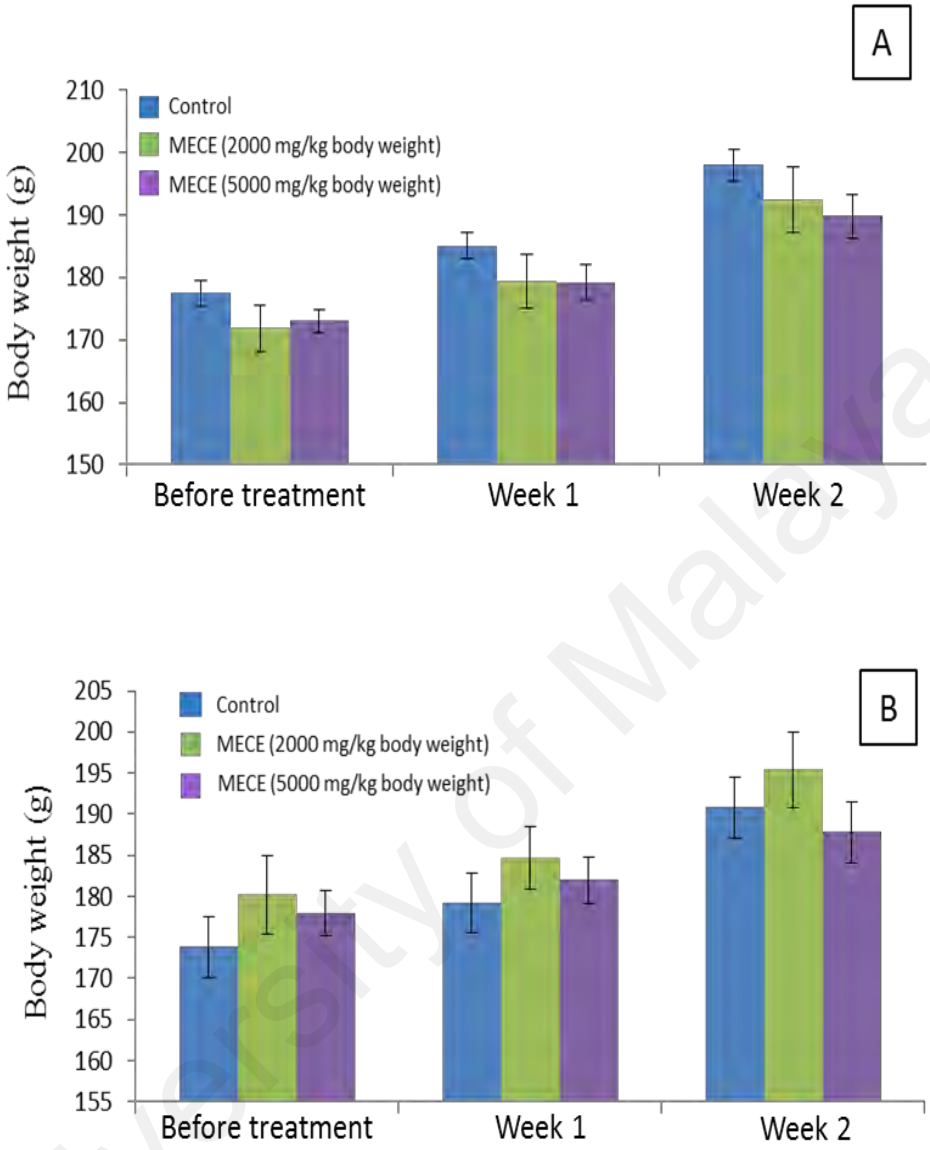

Figure 4.5: Effect of methanolic *C. excavata* leaves extract (MFCE) on body weight of rats. (A) male, (B) female.

Table 4.7: Effect of methanolic extract of *C. excavata* extract on liver biochemical parameters

Treatment Group (MFCE)	ALT(U/I)	AST(U/I)	ALP (U/I)	Albumin (g/dL)	Bilirubin (µmol/L)
Male					
CM	57.0^{a}±2.2	196.0^{a}±3.6	177.7^{a}±7.5	12.2^{a}±0.3	2.19^{a}±0.01
M1(2000mg/kg b.wt)	59.1^{a}±2.3	183.4^{a}±11.7	228.2^{b}27.0	13.0^{b}±0.3	2.73^{b}±0.17
M2(5000mg/kg b.wt)	64.7^{b}±4.3	256.6^{b}±20.4	248.6^{b}±23.4	13.8^{b}±0.3	2.42^{a}±0.22
Female					
CF	46.8^{a}±2.5	165.1^{a}±6.2	143.3^{a}±10.7	14.7^{a}±0.7	1.92^{a}±0.03
F1(2000mg/kg b.wt)	47.8^{a}±2.1	181.6^{a}±15.9	109.0^{b}±5.2	15.2^{a}±0.2	2.76^{b}±0.19
F2(5000mg/kg b.wt)	53.7^{b}±5.2	195.1^{a}±26.4	193.2^{c}±19.3	14.8^{a}±0.3	2.83^{b}±0.16

Values are mean ± SD. ^{a,b,c} For each sex, means within column with different superscript are significant at $p < 0.05$.
ALT = Alanine transaminase; AST = Aspartate transaminase; ALP = Alkaline phosphatase
MFCE = Methanolic extract *Clausena excavata;* CM = Untreated control male; CF = Untreated control female; M1, M2, F1, F2 are treatment groups; b.wt = body weight.

Serum creatinine concentrations in all MFCE male rats were significantly (p<0.05) lower than that of normal control (NC) male rats. However, the mean BUN concentration of female rats treated with 5000 mg/kg body weight was significantly (p<0.05) higher than that of control by 24%. Similarly, serum Na+, K+, and Cl− concentrations were variably elevated among the male and female treatment groups (Table 4.8). The erythrocyte and leukocyte counts and Hb concentration did not vary significantly (p<0.05) between MFCE treatment groups (Table 4.9).

Table 4.8: Effects of methanolic extract of *C. excavata* on kidney biochemical parameters

Treatment (MFCE)	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)	Urea (mmol/L)	Creatinine (umol/L)
Male					
CM	144.31^{a}±7	4.90^{a}±0.10	104.8^{a}±0.1	6.42^{a}±0.34	635.6^{a}±21.4
M1(2000mg/kg b.wt)	145.8^{b}±0.2	4.66^{b}±0.06	103.6^{b}±1.0	4.61^{b}±0.21	379.6^{b}±48.7
M2(5000 mg/kg b.wt)	146.2^{b}±0.4	5.00^{b}±0.25	105.0^{a}±0.4	5.90^{c}±0.21	415.5^{b}±25.7
Female					
CF	144.4^{a}±0.7	4.70^{a}±0.06	104.3^{a}±1.1	5.79^{a}±0.17	576.8^{a}±31.1
F1(2000 mg/kg b.wt)	144.2^{b}±0.7	4.64^{a}±0.29	104.4^{a}±0.4	5.81^{a}±0.24	424.6^{b}±19.3
F2(5000 mg/kg b.wt)	146.1^{b}±0.9	4.90^{a}±0.15	106.8^{b}±0.6	7.70^{b}±0.29	561.6^{a}±29.8

Values are mean ± SD. ^{a,b,c} For each sex, means within column with different superscript are significant at $p < 0.05$. MFCE = Methanolic extract *C.excavata;* CM = Untreated control male; CF = Untreated control female; M1, M2, F1, F2 are treatment groups; b.wt = body weight.

Table 4.9: Effects of methanolic extract of *C. excavata* extract on hematological parameters in rats.

Parameter	Sex	MFCE leaves treatment (mg/kg body weight)		
		Untreated	**2000**	**5000**
Erythrocyte(10^{12}/L)	Male	8.44±0.11	8.04±29.00	8.32±0.20
	Female	7.80±0.03	7.79±0.03	7.77±0.02
Leukocyte (10^{9}/L)	Male	8.46±0.21	9.94±0.37	9.62±0.10
	Female	6.55±0.96	6.20±0.71	6.13±0.34
Hemoglobin (g/L)	Male	13.80±0.20	14.02±0.31	13.40±0.07
	Female	13.10±0.12	12.63±0.48	12.50±0.27

Values are mean ± SD. No significant difference between within or across rows. MFCE = Methanolic extract *Clausena excavata*; Untreated = rats given normal diet without MFCE leaves treatment.

The microscopic evaluation of kidney sections from M1 and M2 rats did not show abnormal histopathological changes. However, kidney sections of F1 and F2 rats showed histopathological changes that varied according to dose level. These changes include mild tubular epithelial degeneration, increased bowman's capsular space as a result of constriction of the glomerulus, mild congestion, cast-like materials within the renal tubular spaces, and mild leukocyte infiltration typified by lymphocyte, and neutrophil infiltration in the renal interstitium (Figure 4.6). Microscopic examination of the liver sections of M1, F1 and M2, F2 rats showed central vein congestion, cloudy hepatocyte swelling, and diffuse infiltration by Kupffer cells. Multifocal areas of mild perilobular necrosis coupled with neutrophil infiltration were observed in some sections of the liver tissue of male rats given a high dose of the extract, indicating that the effect of high dose MFCE leaves was more severe in the male than female rats (Figure 4.7).

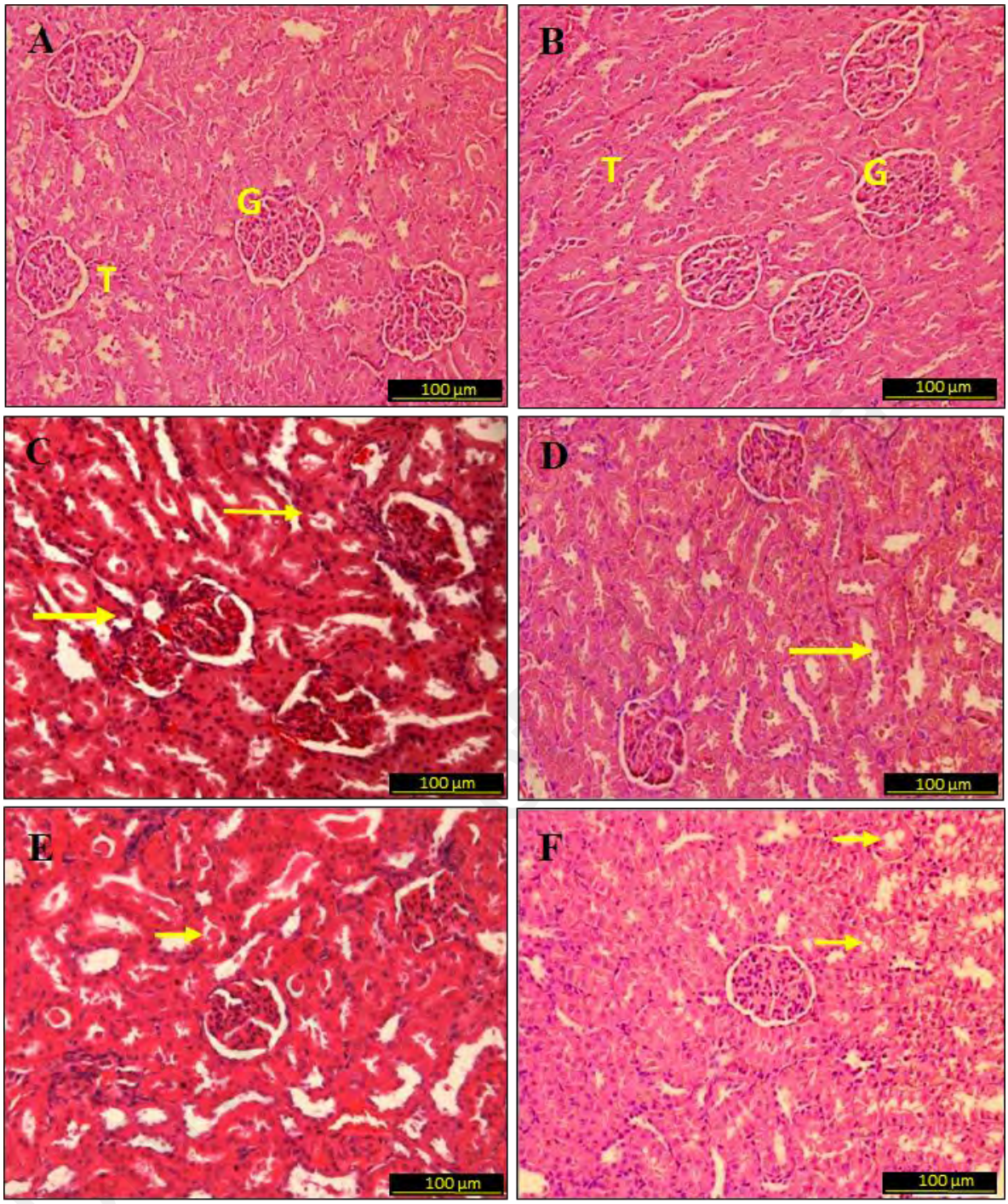

Figure 4.6: Rat kidney tissue after treatment with methanolic *C. excavata* extract. **A & B**: Kidney (control; male and female) showing normal glomeruli (**G**) and tubules (**T**) in the cortical region. **C&D**: Kidney (low dose; 2000 mg/kg, male and female rats) showing tubular degeneration and mild mononuclear cell infiltration (arrows). **E&F**: Kidney (high dose; 5000 mg/kg, male and female rats) with cast in the tubular lumen and mild tubular degeneration (arrows). (H&E) stain.

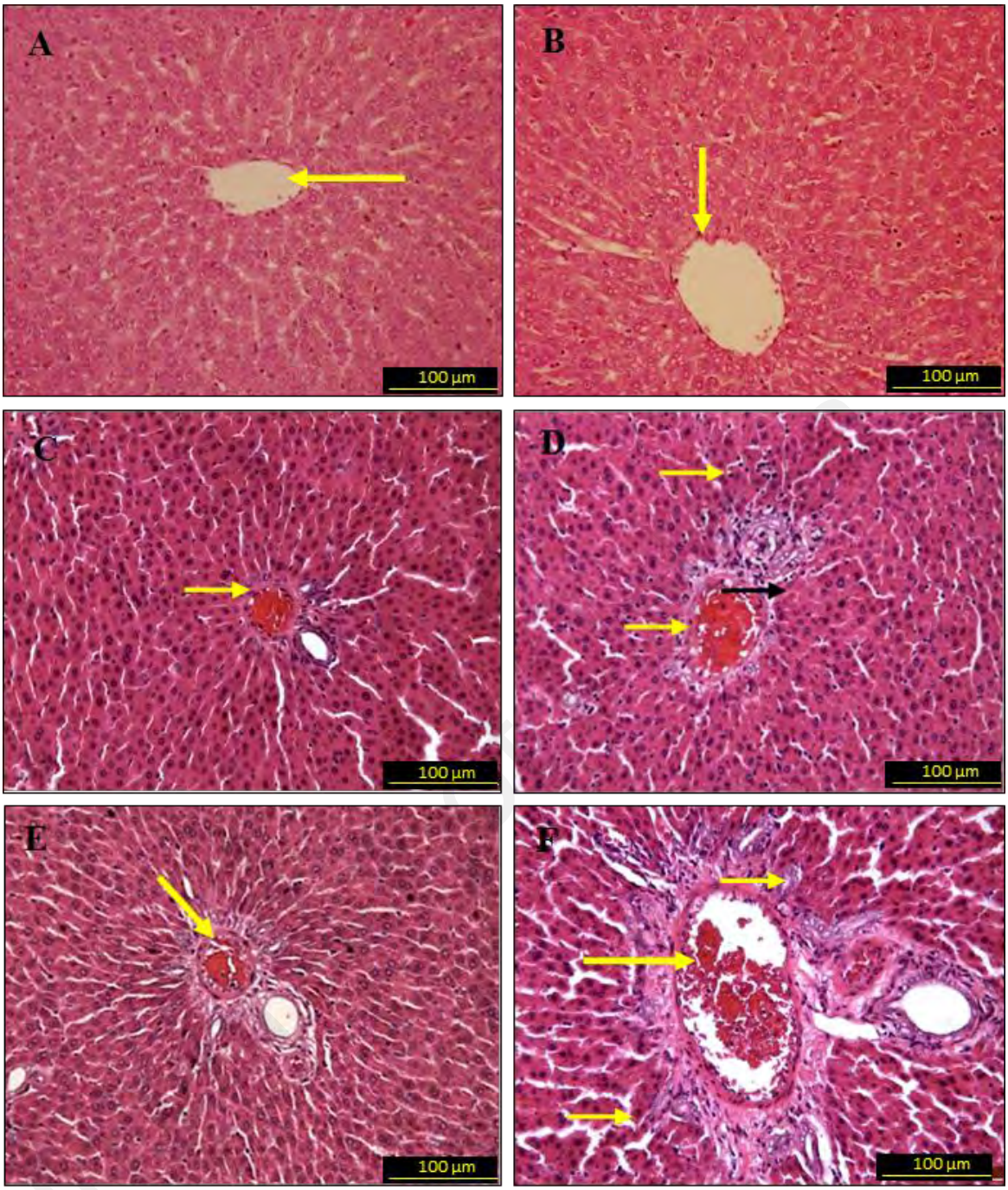

Figure 4.7: Rat liver treated with methanolic *C. excavata* extract. **A & B**:Normal hepatocytes, parenchyma and central vein respectively. **C &D**:Liver (low dose; 2000 mg/kg, male and female rats)showing mononuclear cell infiltrations and congestion of the central vein(arrows). **E& F**: Liver (high dose; 5000 mg/kg, male and female rats) showing infiltration of kupffer cells, vascular congestion and mild periportal necrosis. (H&E) stain.

4.8. Wound healing effect of Methanolic Extract of *C. excavata*

This study was carried out in order to verify the ability of 2% gum acacia containing 50, 100, and 200 mg/mL MFCE to enhance the process of wound healing on rat skin.

4.8.1. Gross wound closure

The gross appearance of wounds at days 5, 10, and 15 post-wound inflictions are presented in (Figure 4.8). The groups treated with various concentrations of MFCE and intrasite gel showed clear signs of dermal healing that was significantly faster and produced much lesser scar compared the group treated gum acacia (Table 4.10). The gross appearance of wound sites was similar among groups at day 5 post-wound infliction. At this point, the difference between the negative control group and the MFCE-treated group was not significant. However, at days 10 and 15, the rate of wound contraction was significantly (p<0.05) better in MFCE-treated than in the negative control group. Progression of wound healing after treatment is shown in Figure 4.8. Although the rate of wound contraction appears to be highest on day 15 (Table 4.10), there was no significant (p>0.05) difference in the rate of healing among the treatment

Table 4.10: Effect of *C. excavata* on wound healing in rats

Group	Treatment	Wound healing post-surgery (%)		
		Day 5	Day 10	Day 15
1	**Gum acacia in normal saline**	21.2±5.1	67.2^{a}±4.3	78.9^{a}±2.5
2	**Intrasite gel (10% CMC)**	20.2±1.3	78.7^{b}±1.2	95.0^{b}±1.8
3	**MFCE (50 mg/mL)**	38.8±2.6	86.7^{b}±1.6	95.7^{b}±0.6
4	**MFCE(100 mg/mL)**	34.5±6.7	84.1^{b}±1.5	97.4^{b}±0.9
5	**MFCE(200 mg/mL)**	34.8±4.5	82.9^{b}±2.0	98.8^{b}±0.4

All values were expressed as mean and ±standard error mean.^{a,b}Means with different superscripts within column are significantly different ($P<0.05$). MFCE: Methanol extract of *Clausena excavate*
groups.

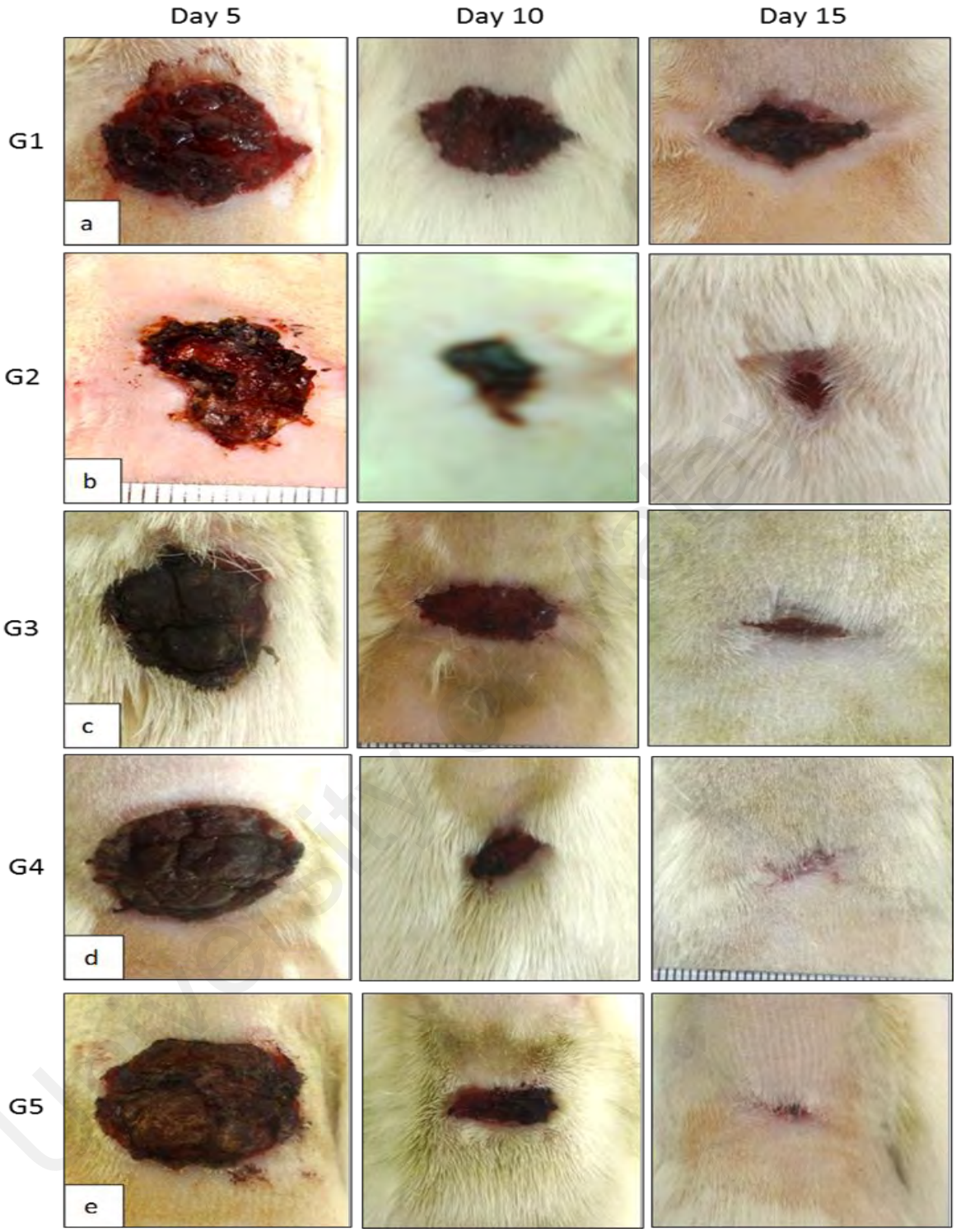

Figure 4.8: Gross skin lesion showing wound healing after treatment for 5, 10, and 15 days. (G1) gum acacia-treated group, showing incomplete wound healing (G2) intrasite (10% CMC) gel group (G3) MFCE-LD (50mg/mL) group (G4) MFCE-MD (100mg/mL) group and (G5) MFCE-HD (200 mg/mL) group. Wound healing and contraction is improves with increase in MFCE concentration.

4.8.2. Histopathological assessment of wound healing tissue

4.8.2.1. Hematoxylin and eosin-stained sections

Hematoxylin and Eosin was performed to assess basic histopathological changes. The histopathology of tissue sections on day 15 post-wound infliction was evaluated by an observer blinded to the experimental protocol (Table 4.11). There was significantly ($P<0.05$) greater epithelialization in the epidermis of the treatment groups than in that of the control group. In general, more inflammatory cells, degeneration, and granulation tissue were observed in the skin of the control group than in that of the treatment groups ($P<0.05$), suggesting enhanced healing in response to MFCE treatment (Figure 4.9 and Table 4.11).

4.8.2.2. Masson' trichome -stained sections (MT)

Masson trichrome staining was used to detect collagen fibers in the healed skin area. The changes in Masson trichome-stained skin tissues are presented in Table 4.12. The color intensity of the stained tissues was significantly ($P<0.05$) greater in the MFCE-HD group than in the other groups. There was significantly ($P=0.0003$) lesser distribution of fibroblasts in the control group than in the other treatment groups. Collagen maturity was higher ($P=0.0009$) in the MFCE-HD and intrasite groups, while the rates of angiogenesis ($P=0.0121$) and collagen distribution ($P=0.0041$) were significantly ($P< 0.05$) higher in the MFCE-MD and MFCE-HD compared with the other groups (Figure 4.10 and Table 4.12).

Table 4.11: Histopathological evaluation of the epidermis and dermis of H&E-stained skin sections of *C. excavata* methanol extract-treated rats

Parameters	Lesion Score (Unit)				
	Control	MFCE-LD	MFCE-MD	MFCE-HD	Intrasite
Epidermis					
Epithelialization	0.5^{a}±0.2	4.0^{b}±0.0	4.0^{b}±0.0	4.0^{b}±0.0	4.0^{b}±0.0
Inflammatory Cell	2.5^{a}±0.6	0.3^{bc}±0.2	1.7^{ac}±0.2	0.5^{bc}±0.2	1.5^{ac}±0.2
Degeneration	2.3^{a}±0.5	0.5^{b}±0.2	0.5^{b}±0.2	0.7^{b}±0.2	2.3^{a}±0.2
Dermis					
Granulation tissue	2.7^{a}±0.3	1.3^{b}±0.2	1.8^{ab}±0.3	1.5^{b}±0.3	1.8^{ab}±0.2
Inflammatory cell	2.5^{a}±0.2	1.5^{bc}±0.2	1.2^{b}±0.2	1.0^{b}±0.0	2.1^{ac}±0.2

All values were expressed as mean and ±standard error mean. Mean with different superscripts were significantly different (P<0.05). MFCE: Methanol fraction *Clausena excavata*; LD: Low dose (50 mg/mL); MD: Medium dose (100 mg/mL); HD: High dose (200 mg/mL), intrasite (10% CMC)

Table 4.12: Evaluation of connective tissue and vascular proliferation in Masson's Trichome-stained skin sections of *C. excavata* methanol extract-treated rats.

Parameter	Lesion Score (Unit)				
	Control	MFCE-LD	MFCE-MD	MFCE-HD	Intrasite
Intensity of coloration	1.6^{ab}±0.2	1.5^{ab}±0.2	2.3^{ac}±0.2	2.7^{c}±0.2	1.2^{b}±0.2
Fibroblast distribution	1.5^{a}±0.2	2.7^{b}±0.2	3.3^{b}±0.2	3.5^{b}±0.2	2.8^{b}±0.2
Collagen maturity	1.3^{a}±0.2	1.5^{a}±0.2	2.0^{ab}±0.0	3.3^{c}±0.4	2.5^{bc}±0.2
Angiogenesis rate	2.0^{a} ±0.4	2.3^{ab}±0.2	3.5^{c}±0.2	3.1^{bc}±0.2	2.7^{a}±0.2
Collagen distribution	2.1^{a}±0.6	2.2^{a}±0.2	3.7^{bc}±0.2	4.0^{bc}±0.0	2.8^{ac}±0.2

All values were expressed as mean ±standard error mean. Mean with different superscripts were significantly different (P<0.05). MFCE: Methanol fraction *Clausena excavata*; LD: Low dose (50 mg/mL); MD: Medium dose (100 mg/mL); HD: High dose (200 mg/mL), intrasite (10% CMC)

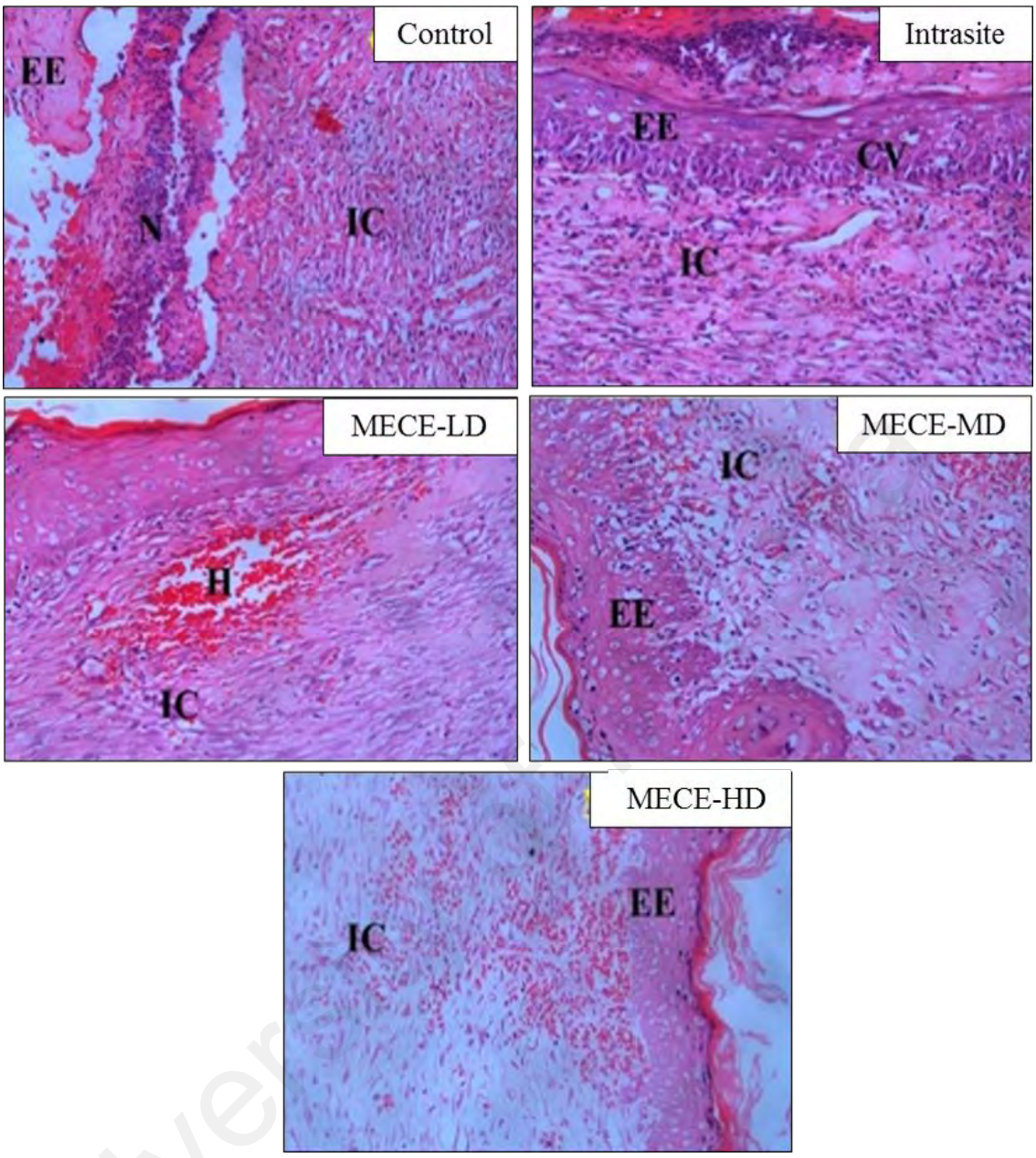

Figure 4.9: Skin of rats treated with methanol *Clausena excavata* leaves extract (MFCE) (Hematoxylin and Eosin staining). Skin sections of the control show incomplete epidermal epithelialization (EE) with necrosis (N) and abundant inflammatory cell response (IC) in the dermis; intrasite-treated skin showed complete EE with cellular vacuolation (CV) and light scattering of inflammatory cells (IC) in the dermis; low dose (MFCE-LD) (50 mg/mL) showed incomplete EE and a focal hemorrhage (H) in the dermis; medium dose (MFCE-MD) (100 mg/mL) showed complete EE with occasional evidence of IC in the dermis; and high dose (MFCE-HD) (200 mg/mL) showed complete EE and occasional evidence of IC in the dermis. (H&E, 200×).

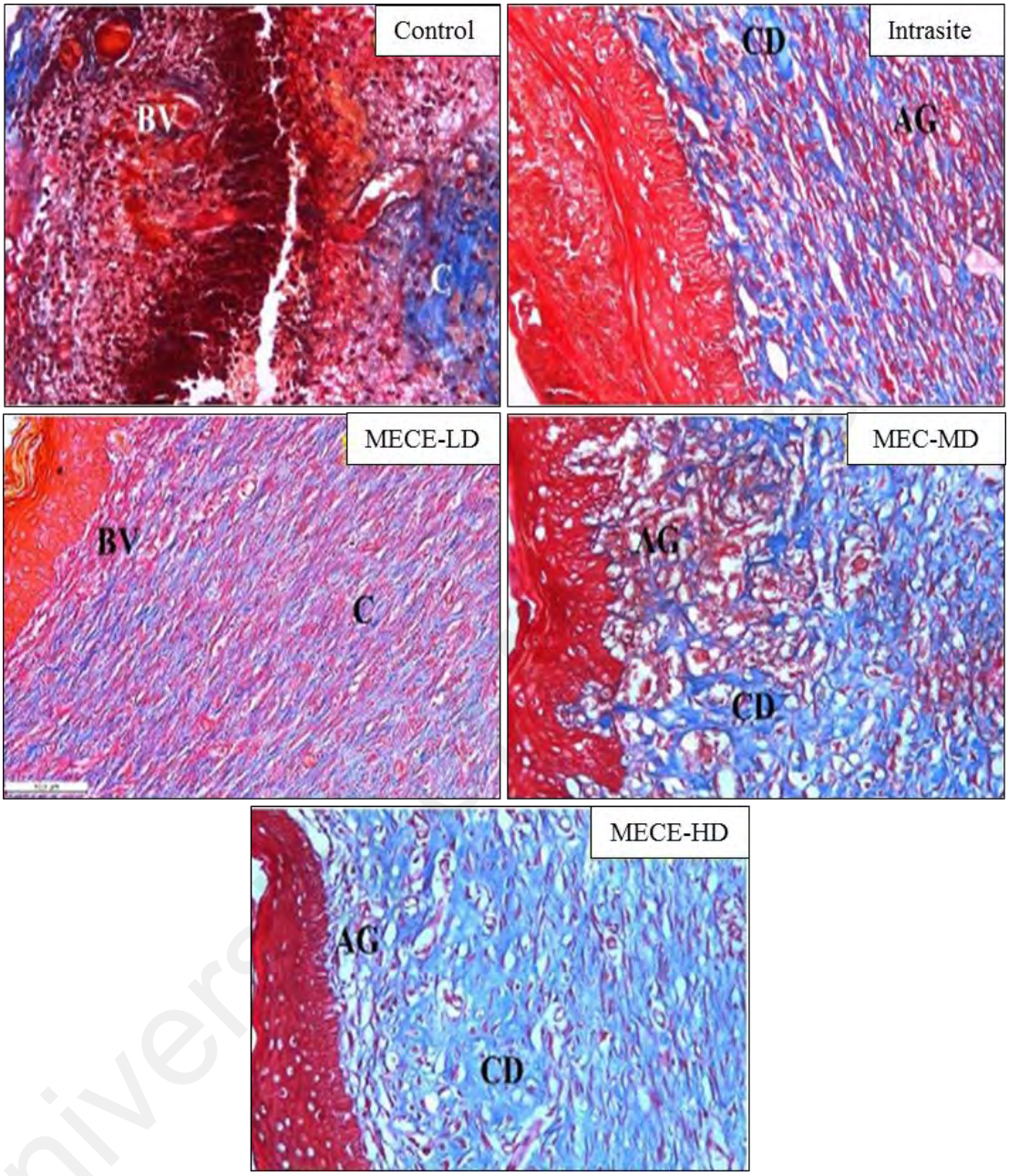

Figure 4.10: Rat skin treated with methanol *Clausena excavata* leaves (Masson's trichome stain). Sections of control tissue showing occasional light scattering of collagen (C) and blood vessels (BV) in the dermis; Intrasite treatment with angiogenesis (AG) and collagen (CD); low dose (MFCE-LD) (50 mg/mL) with light scattering of collagen (C) and abundant blood vessels (BV) in the dermis; medium dose (MFCE-MD)(100 mg/mL) abundant angiogenesis (AG) and collagen (CD); high dose (MFCE)(200mg/mL) showing abundant angiogenesis (AG) and confluent collagen (CD)(200x).

4.8.2.3. Immunohistochemical analysis of VEGF and TGF-β

Immunohistochemistry was performed to show the expression of VEGF and TGF-β1 in the skin wounds of control and treated groups. The number of VEGF-positive cells was significantly (*P<0.05*) lower in the control and MFCE-LD groups than in either the MFCE-HD, MFCE-MD, or intrasite groups (Figure 4.11a & b). Extracellular matrix deposition determined by the number of TGF-β1-positive cells was significantly (*P*<0.05) higher in tissues treated with MFCE and intrasite gel than the untreated control (Figure 4.12a & b).

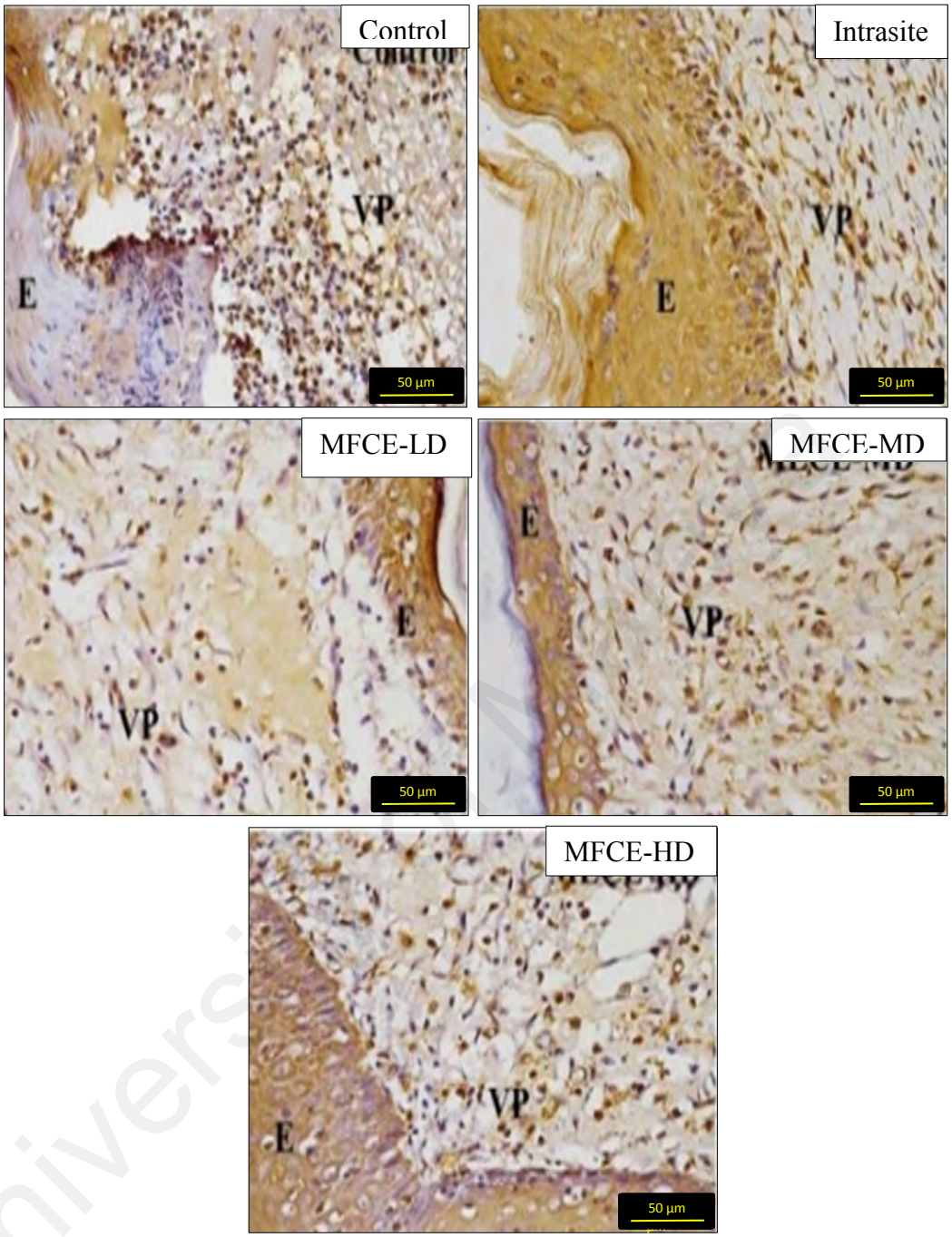

Figure 4.11a: Immunochemical analysis for vascular epidermal growth factor (VEGF) expression in rat skin treated with methanol *C. excavata* extract (MFCE). Skin section with epidermis (E) showing distribution of light to dark brown VEGF-positive cells (VP) in the untreated control, intrasite, low dose (MFCE-LD = 50 mg/mL), medim dose (MFCE-MD = 100 mg/mL) and high dose (MFCE-HD = 200 mg/mL) treatment groups (Immunoperoxidase).

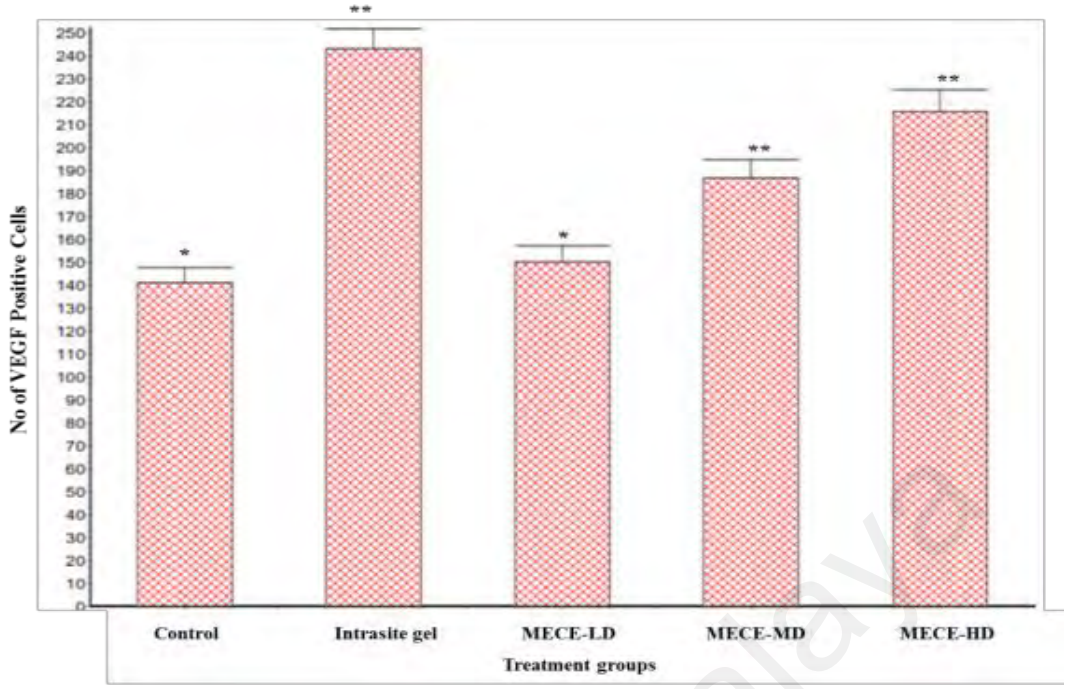

Figure 4.11b: Quantitative estimation for vascular epidermal growth factor (VEGF) protein expression in rat skin treated with methanol *C. excavata* extract (MFCE). Bar graph showing distribution of VEGF-positive cells in the Control, Intrasite, and MFCE-treated rat skin wound. *Indicates significance differences at $P<0.05$, ** Indicates not significant within groups. Control = untreated, low dose (MFCE-LD = 50 mg/mL), medium dose (MFCE-MD = 100 mg/mL), high dose (MFCE-HD = 200 mg/mL), and Intrasite = (10% CMC) (Immunoperoxidase, 400×).

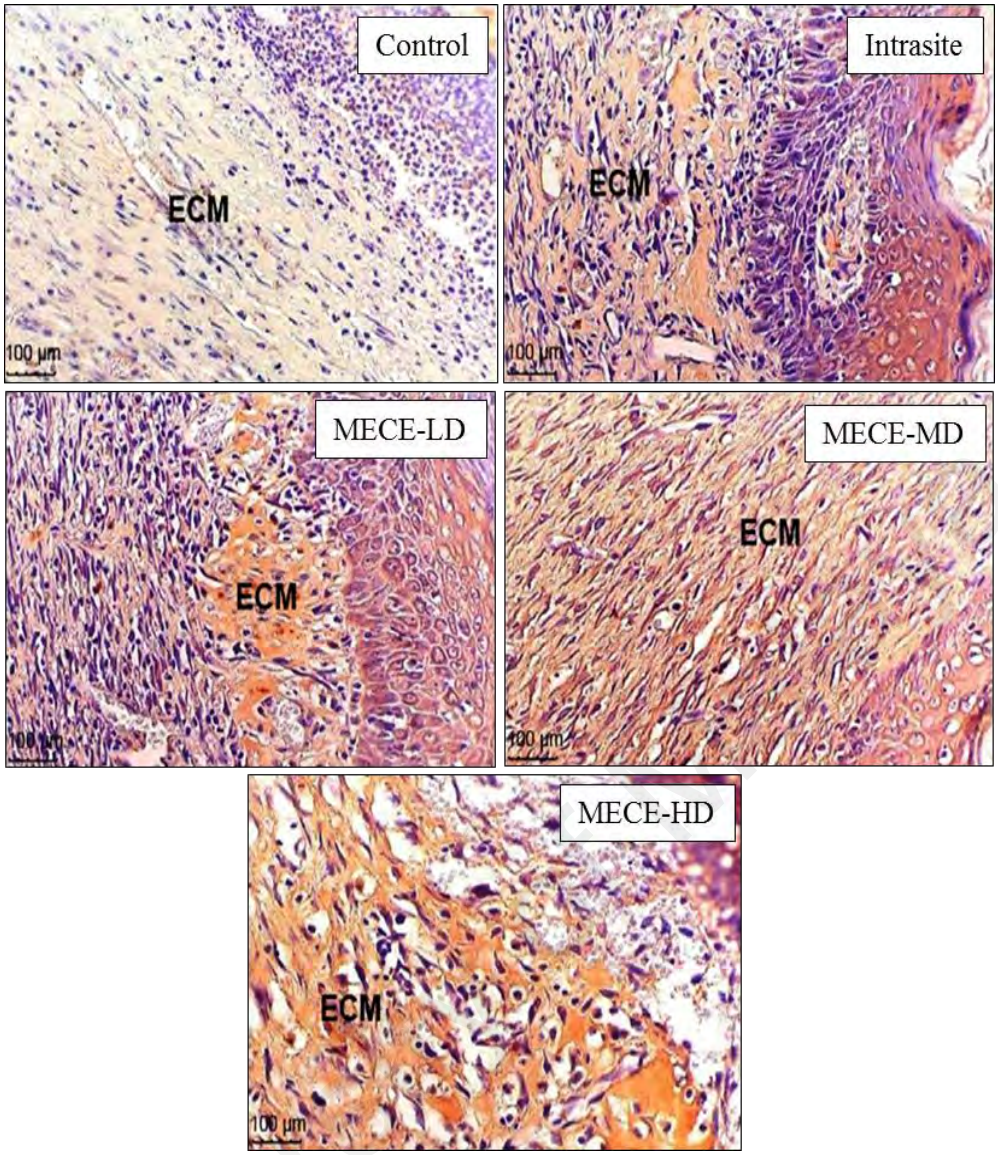

Figure 4.12a: Quantitative estimation for transforming growth factor-β1 (TGF-β1) expression in rat skin treated with methanol *Clausena exca*v*ata* extract (MFCE). Control: showing little deposition of extracellular matrix (ECM), Intrasite (10% CMC): moderate ECM deposition, MFCE-LD (50 mg/mL): little to moderate ECM deposition, MFCE-MD (100 mg/mL): moderate ECM deposition, MFCE-HD (200 mg/mL): confluent ECM deposition. (Immunoperoxidase, 200×).

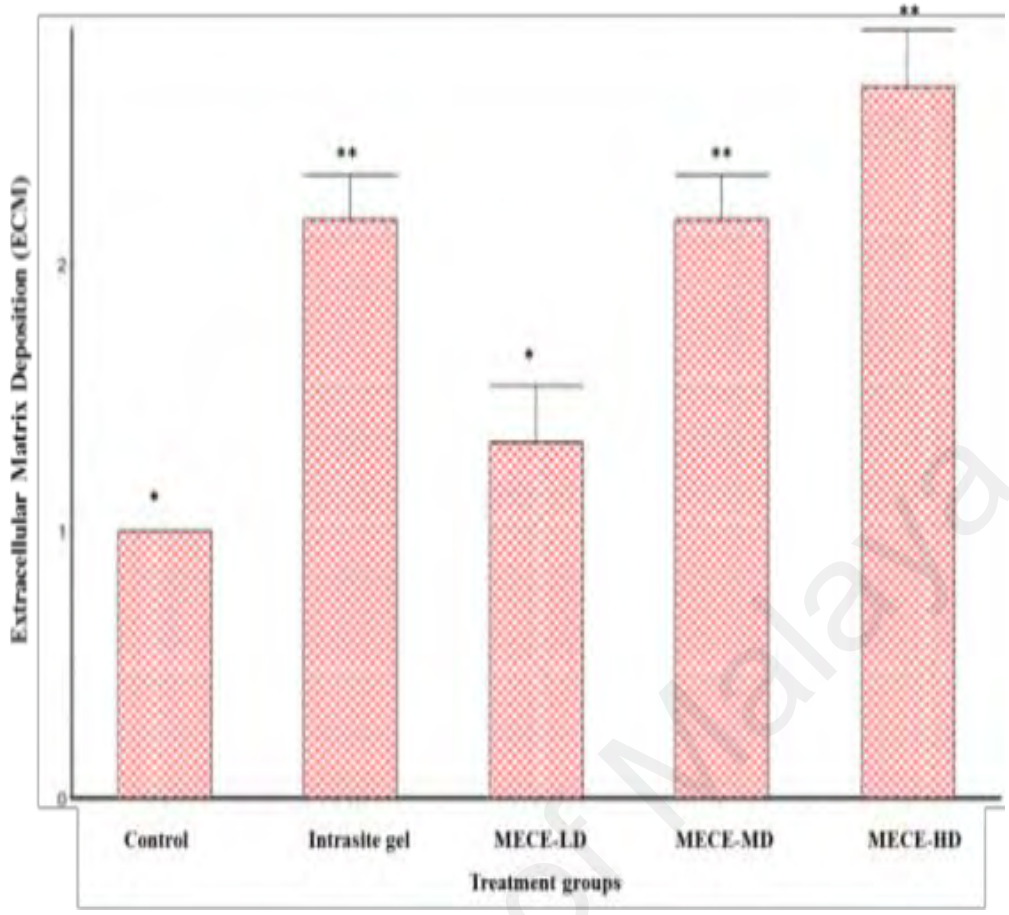

Figure 4.12b: Quantitative estimation for transforming growth factor-β1 (TBG-β1) protein expression in rat skin treated with methanol *C. excavata* leaf extract (MFCE). Bar graph showing distribution of extracellular matrix (ECM) in TGF-β1-stained skin sections of control, intrasite gel (10% CMC), and MFCE-treated rat skin wound. *Indicates significance differences at $P<0.05$. The extracellular matrix distribution (ECM) was evaluated by using a four-point scale; 0 (no ECM); 0%, 1 (little ECM); 30%, 2 (moderate ECM); 65% and 3 (confluent ECM); 70%–100.

4.8.3. Antioxidant enzymes SOD, CAT, GPx and LPO evaluation in the healed wound area

Antioxidant enzymes, SOD, CAT and Gpx and LPO were estimated in this study to determine the progression of the wound healing and to determine the effect of MFCE. The results reveal that the SOD activity was significantly (P<0.05) higher in the MFCE-HD (4.57±0.23 U/mL), MFCE-MD (5.39±1.01 U/mL), and intrasite gel groups (4.91±0.8 U/mL) than the control (1.83±0.2 U/mL). There was no significant difference between treatment groups (Figure 4.13A). The CAT activity was significantly (P<0.05) higher in the MFCE-HD (28.55±4.1 nmol/mL) and intrasite gel groups (26.90±1.79 nmol/mL) than that in the control group (17.82±1.07 nmol/mL) (Figure 4.13B). In addition, there was an increase in the MFCE-MD (23.77±1.40 mmol/mL) and MFCE-LD (20.91±0.90 nmol/mL) groups but it was not significant. The GPx activity was significantly (P<0.05) higher in the intrasite (32.9±1.87 nmol/mL), MFCE-MD (29.33±1.52 nmol/mL), and MFCE-HD (23.0±2.5 nmol/mL) than in the control (16.4±0.35 nmol/mL) or MFCE-LD (21.8±2.02 nmol/mL) groups (Figure 4.13C). The LPO activity was significantly (P<0.05) higher in the control group (9.12±1.76 μM) than in the intrasite (2.81±0.94 μM), MFCE-HD (4.17±1.10 μM), MFCE-MD (4.85±0.75 μM), or MFCE-LD (4.09±0.76 μM) groups (Figure 4.13D).

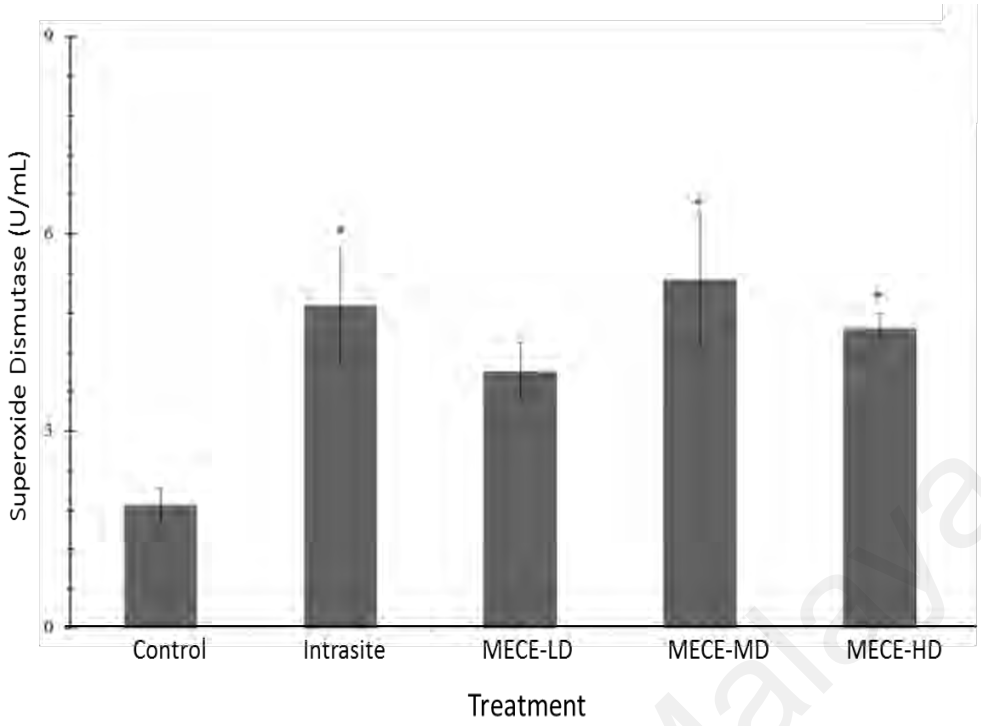

Figure 4.13a: Methanol *Clausena excavata* extract (MFCE) treatment rat wound superoxide dismutase (SOD) activity. Treatment with intrasite, MFCE-MD and MFCE-HD produced higher SOD activites in the wound than control. Values are presented as mean ± S.D of 6 rats/group. *Significant ($P< 0.05$) compared to control. Control = untreated, Intrasite = (10% CMC), low dose (MFCE-LD; 50 mg/mL), medium dose (MFCE-MD; 100 mg/mL), high dose (MFCE-HD; 200 mg/mL).

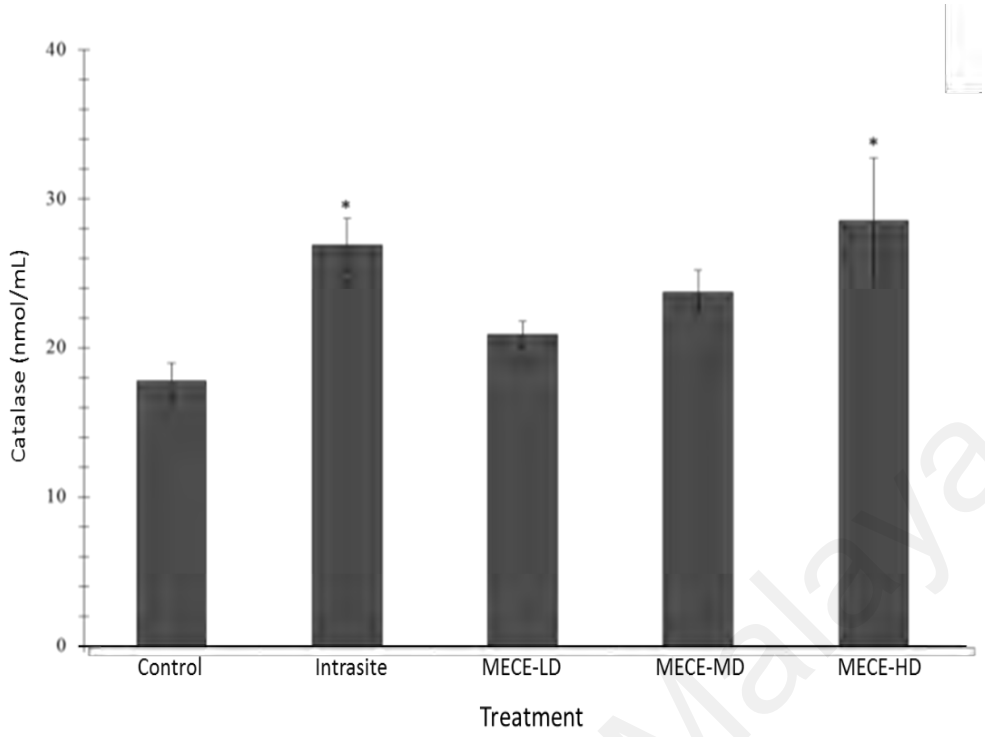

Figure 4.13b: Methanol *Clausena excavate* extract (MFCE) treatment rat wound catalase (CAT) concentration. Treatment with intrasite and MFCE-HD produced higher CAT concentration in the wound than control. Values are presented as mean ± S.D of 6 rats/group. * Significant ($P< 0.05$) compared to control. Control = untreated, Intrasite = (10% CMC), low dose (MFCE-LD; 50 mg/mL), medium dose (MFCE-MD; 200 mg/mL), high dose (MFCE-HD; 100 mg/mL).

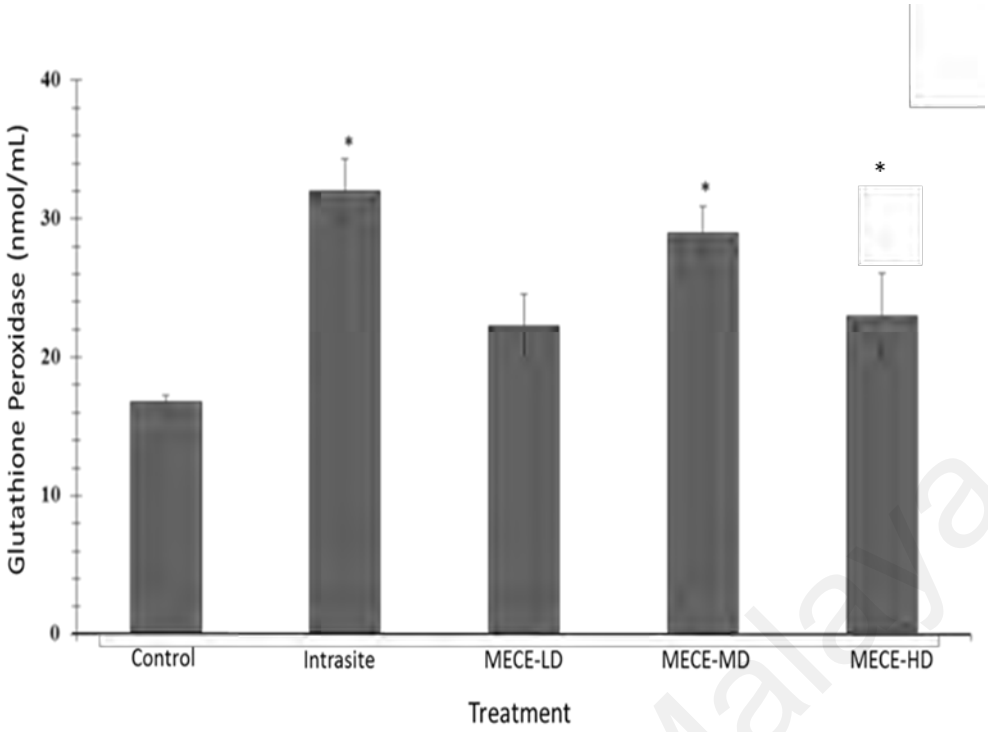

Figure 4.13c: Methanol *Clausena excavata* leaf extract (MFCE) treatment rat wound glutathione peroxides (GPx) concentration. Treatment with intrasite, MFCE-MD and MFCE-HD produced higher GPx concentration in the wound than control. Values are presented as mean ± S.D of 6 rats/group. * Significant ($P< 0.05$) compared to control. Control = untreated, Intrasite = (10% CMC), low dose (MFCE-LD; 50 mg/mL), medium dose (MFCE-MD; 100 mg/mL), high dose (MFCE-HD; 200 mg/mL).

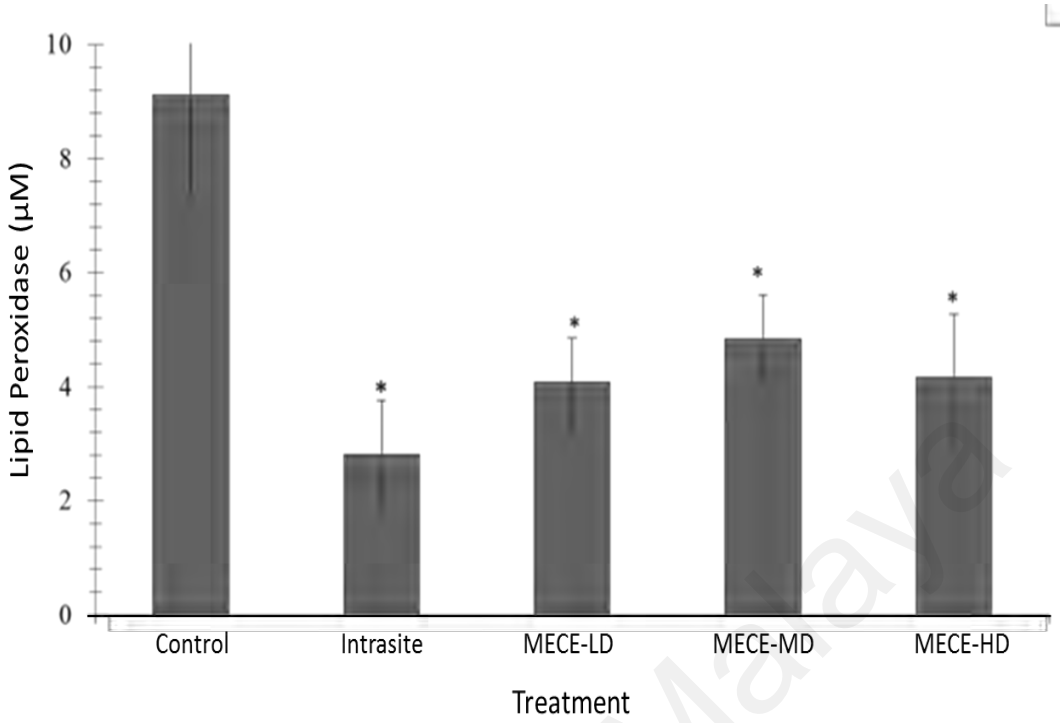

Figure 4.13d: Methanol *Clausena excavata* extract (MFCE) treatment rat wound lipid peroxidase (LPO) quantity. All treatments produced lower LPO quantity in the wound than control. Values are presented as mean ± S.D of 6 rats/group. * Significant ($P<$ 0.05) compared to control. Control = untreated, Intrasite = (10% CMC), low dose (MFCE-LD; 50 mg/mL), medium dos (MFCE-MD; 100 mg/mL), high dose (MFCE-HD; 200 mg/mL).

4.8.4. Estimation of TNF-α -6 and IL-10 in the healed wound area

Since inflammation plays a vital role in the pathogenesis of impaired wound healing, the down regulation of these proinflammatory cytokines by the methanolic fraction of *C. excaavata* is important in the regulation of prolong inflammatory response. The results show that the level of TNF-α and IL-6 were significantly (P<0.05) lower in the group treated with 100 and 200 mg/mL MFCE than in the untreated (Figure 4.14 a&b). By contrast, the level of IL-10 was significantly ($P<0$.05) higher in the 200 mg/mL MFCE-treated group compared to control (Figure 4.14c).

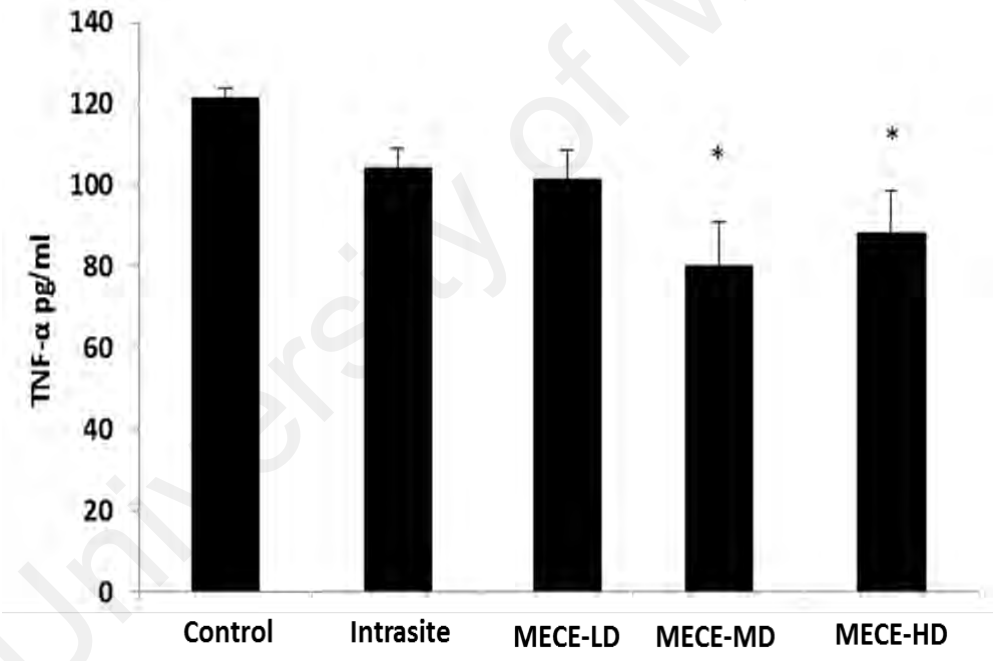

Figure 4.14a: TNF-α level in wound tissue of rats treated with methanol *C. excavata* extract (MFCE). The TNF-α level were significantly lower in MFCE-MD and MFCE-HD-treated than in control. MFCE-LD = 50 mg/mL; MFCE-MD = 100 mg/mL; MFCE-HD = 200 mg/mL. All values are presented as mean ± S.D of 6 rats/group. *Significant difference at P < 0.05.

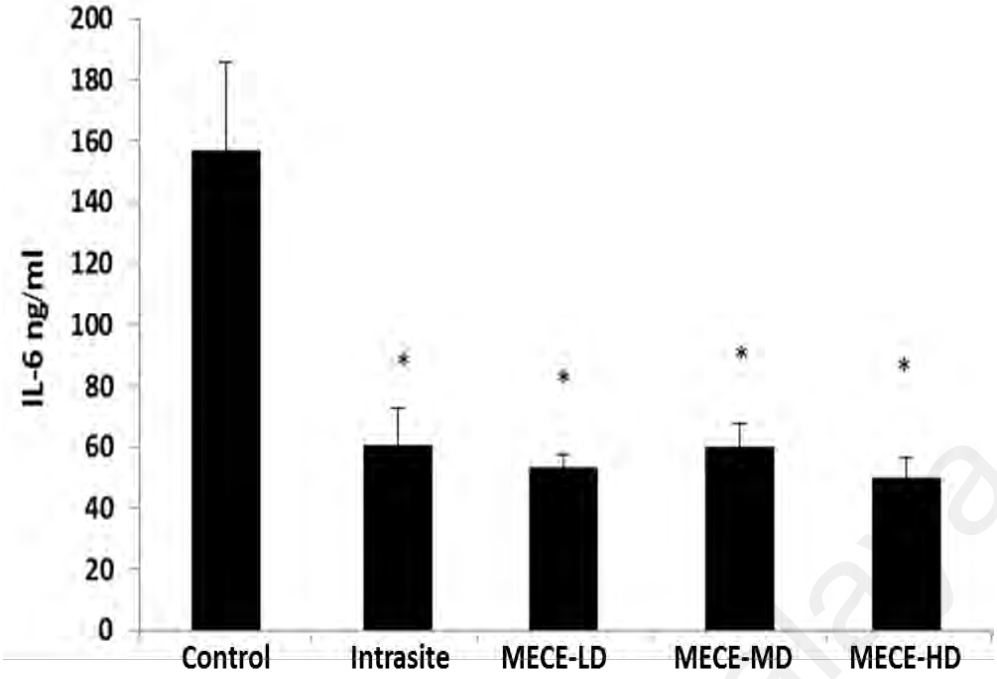

Figure 4.14b: IL-6 level in wound tissue of rats treated with methanol *Clausena excavata* extract (MFCE). The IL-6 levels were significantly lower in all treatment groups compared to untreated control. MFCE-LD = 50 mg/mL; MFCE-MD = 100 mg/mL; MFCE-LD = 200 mg/mL. All values are presented as mean ± S.D of 6 rats/group. * Significant difference at $P < 0.05$.

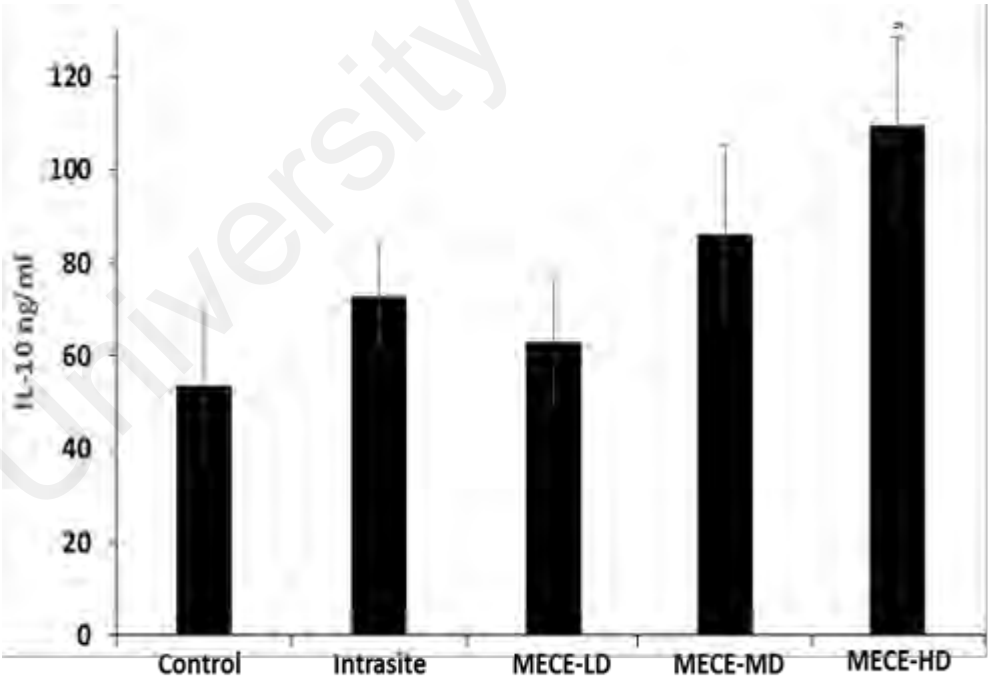

Figure 4.14c: IL-10 level in wound tissue of rats treated with methanol *Clausena excavata* extract (MFCE). No significant difference among MFCE treatment groups. MFCE-LD = 50 mg/mL; MFCE-MD = 100 mg/mL; MFCE-HD = 200 mg/mL. All values are presented as mean ± S.D of 6 rats/group. * Significant difference at $P < 0.05$ compare to control.

4.8.5. Gene expression

Immortality of inflammatory phase could generate overproduction of ROS at wound site results in the induction of apoptosis, which might be a crucial reason for delay wound healing. Therefore BAXand BCL2, which play an important role in apoptosis process were choosen in present study.

Cox-2 is reported to be expressed during inflammation phase and it is found to be induced in macrophage by proinflammatory cytokines. Thus, demonstrating that the wound-healing process was at a later stage, and enter remodeling phase as a part of return to the normal situation by investigation these genes.

The RNA concentration, purity and integrity in the wounded rat skin sample were measured by Nano Drop ND-2000 Spectrophotometer and gel electrophoresis are shown in APPENDIX C.

The optimal annealing temperature chosen for reference and target genes was based on the lowest Cq values in the gene expression assay, as well as the melting peak (Figure 4.15). The standard curve shown for both reference and target genes based on the cDNA serial dilution (Figure 4.16). All genes showed efficiency of between 96 and 108%. The expression level of the target gene was normalized with two endogenous housekeeping genes, the β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The C_T value of the target gene showed that there are differences in gene expression between the calibrator group (vehicle control group) and treated groups. From the results, the relative mRNA expression level of BAX gene was down-regulated in the HD-MFCEtreated group but not significantly (Figure 4.17). On the other hand, there was a significant ($P<0.05$) up-regulation in the BCL2 gene in the MFCE-treated group (Figure 4.17b). The relative expression of COX-2 was significantly ($P<0.05$) down-regulation in MFCE-treated group (Figure 4.17 c).

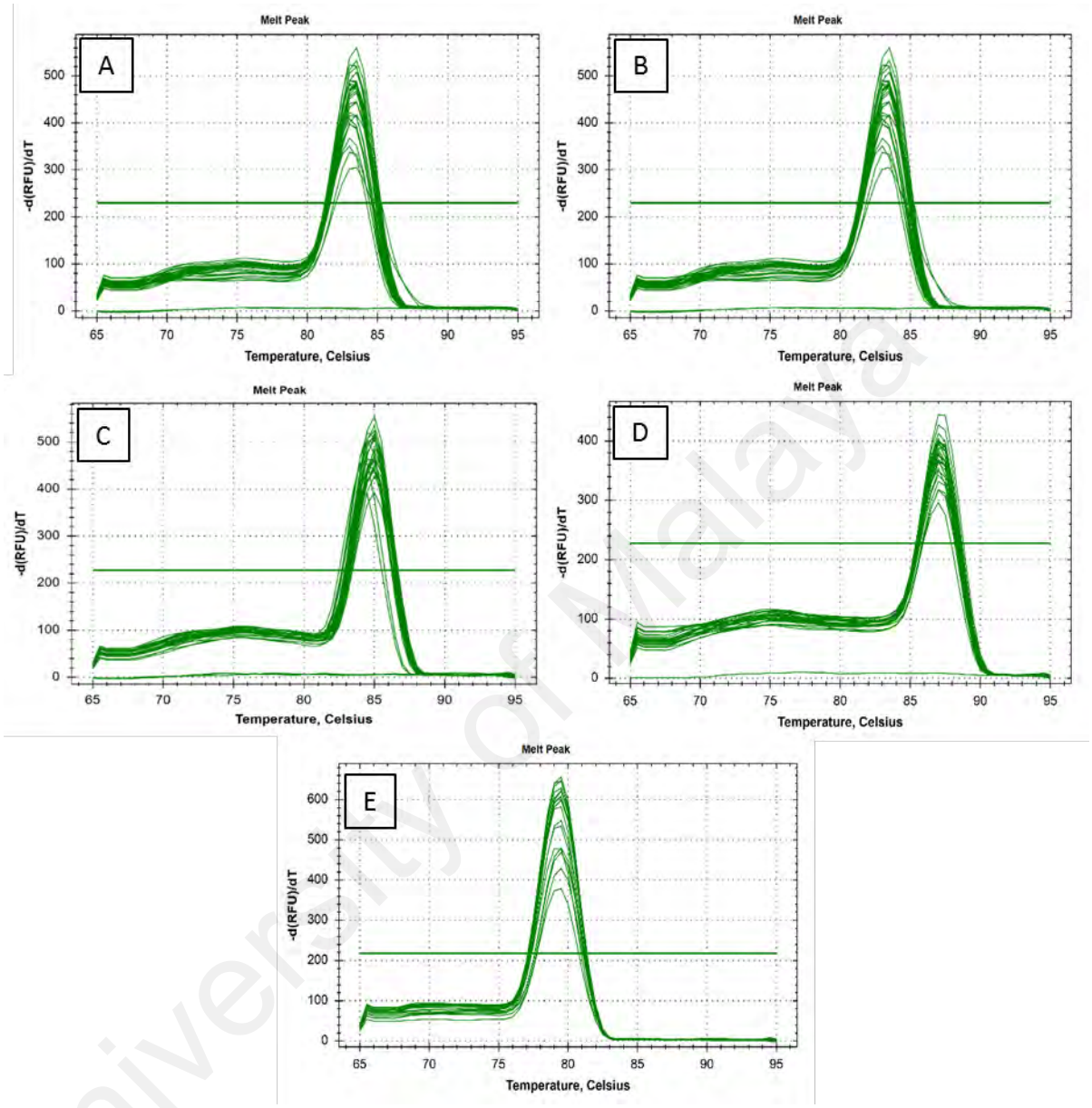

Figure 4.15: Quantitative real-time PCR melting curves for (A) β-actin, (B) glyceraldehyde 3-phosphate dehydrogenase (GAPDH), (C) BAX, (D) Bcl-2, and (E) COX-2 genes in rat skin wound, analyzed by CFX manager ^{TM} software.

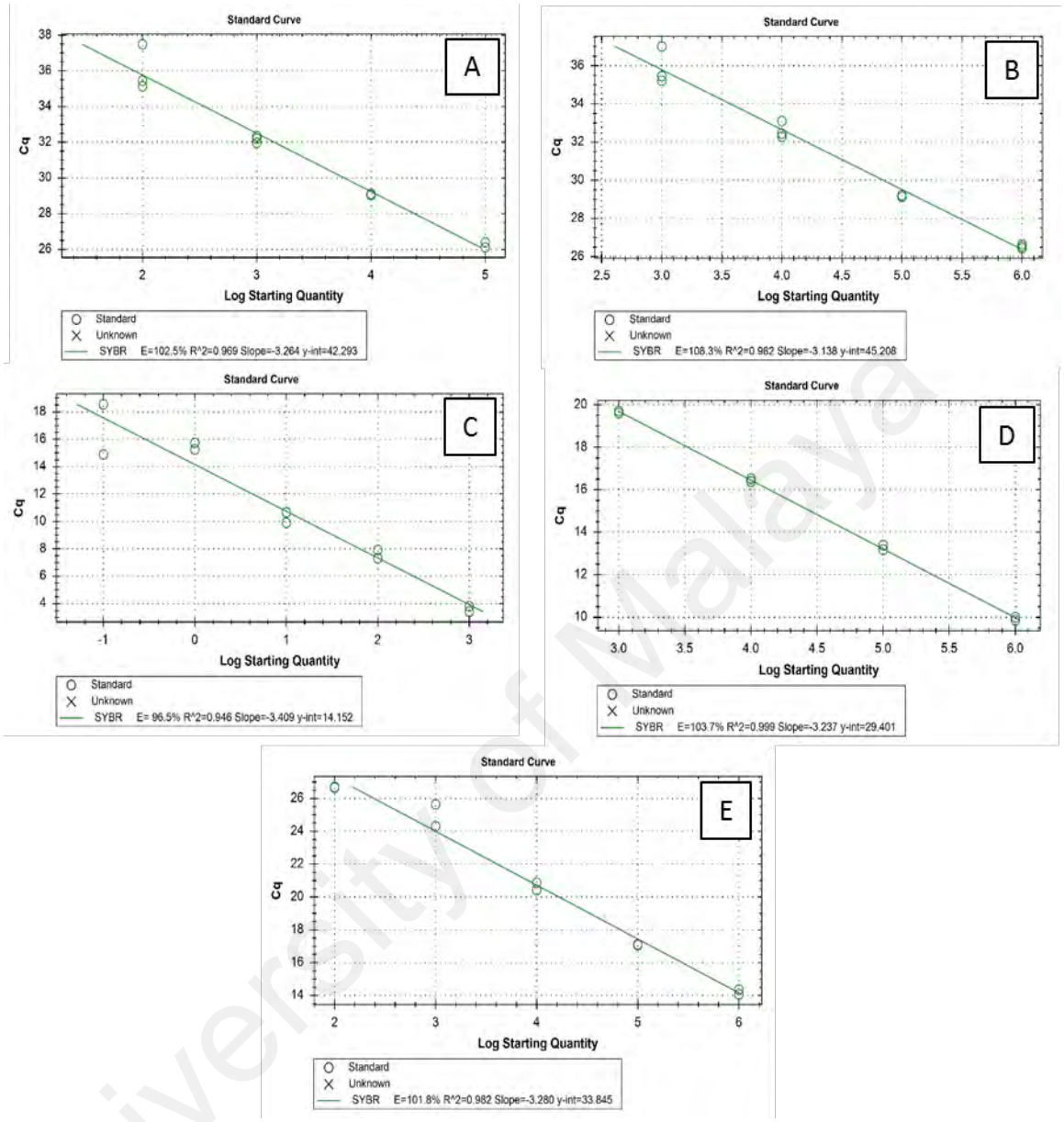

Figure 4.16: Quantitative real-time PCR standard curve for (A) β-actin, (B) glyceraldehyde 3-phosphate dehydrogenase (GAPDH), (C) BAX, (D) Bcl-2, and (E) COX-2 genes in rat skin wound, analyzed by CFX manager TM software.

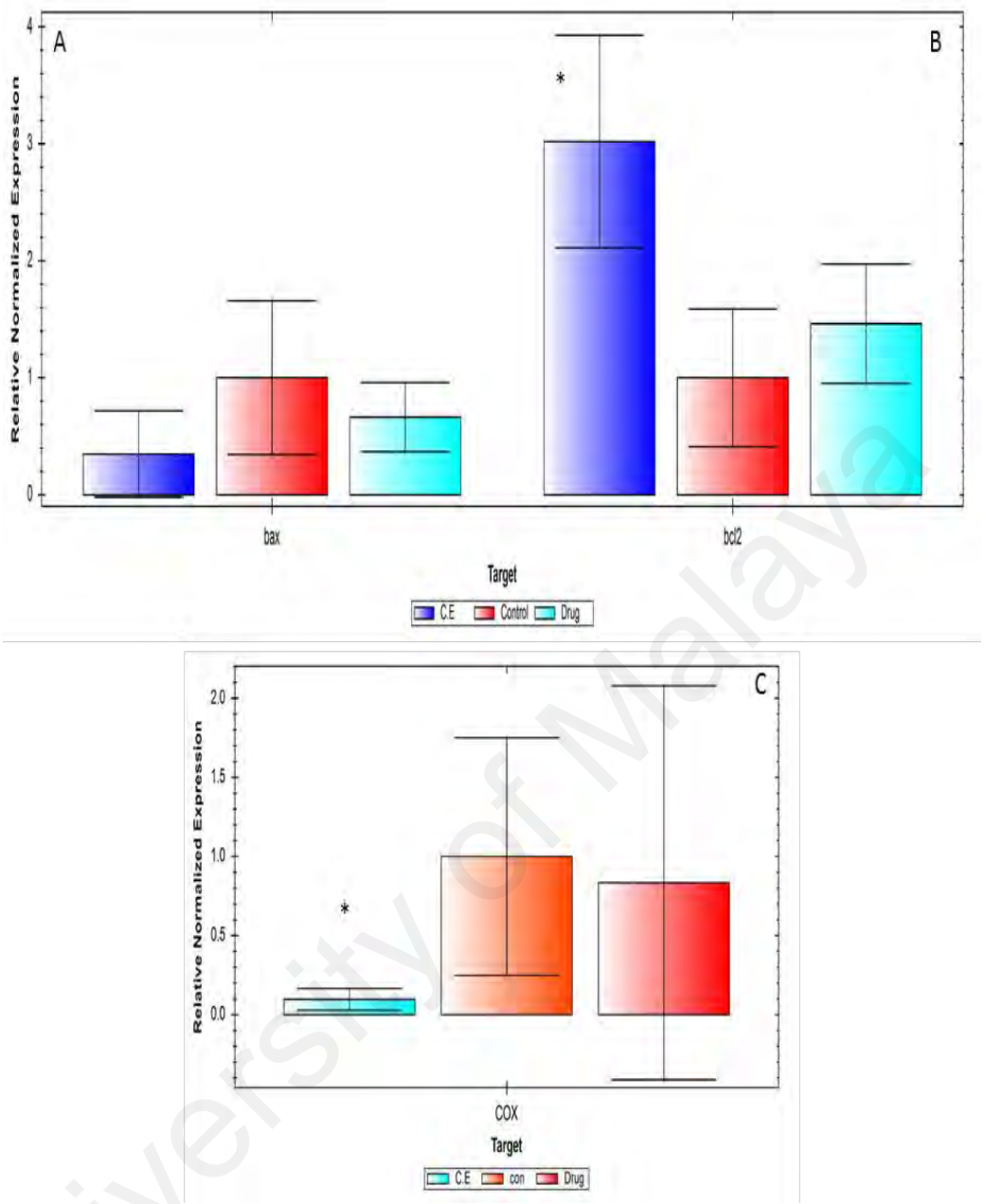

Figure 4.17: Relative expression of BAX, BCL2 and COX-2 genes in the skin wounds of rats treated with 200 mg/mL methanolic *Clausena excavata* extract (MFCE). Relative gene expression levels were calculated using the delta-delta C_T method and values are presented as mean ± SD, (5 rats/group) *significantly different at $P<0.05$ versus calibrator (vehicle control group). Control = untreated; Reference drug = Intrasite gel.

4.9. Gastroprotection effect of methanolic extract *Clausena excavata*

4.9.1. Pyloric ligation model

Treatment with MFCE at 200 and 400 mg/kg and omeprazole at 20 mg/kg body weight significantly ($p < 0.05$) reduced the volume and total acidity of gastric juice at 4 h post-treatment (Table 4.13). Furthermore, rats pre-treated with omeprazole and MFCE in all the doses showed significantly ($p<0.05$) higher production of gastric mucus than the untreated control (Figure 4.18).

Table 4.13: Effect of methanolic *C. excavata* extract treatment on gastric juice parameters of rats with gastric ulcer.

Treatment	Volume of Gastric juice (mL)	pH of Gastric juice	Total acidity (mEq/L)
5%Tween 20 (Normal control)	5.15±0.5	1.46±0.13	3717.90±146.70
Omeprazole (20mg/kg Bwt)	2.22*±0.30	3.82*±0.10	1976.65*±389.02
MFCE (200 mg/kg Bwt)	2.62*±0.21	2.59*±0.11	1578.98*±235.95
MFCE (400 mg/kg Bwt)	1.73*±0.27	2.73*±0.31	1102.37*±82.20

All the values are expressed as the mean ± SEM. *Significant differences at ($p<0.05$) compared with normal control. MFCE = methanolic *C. excavata* extract. Bwt = Body weight.

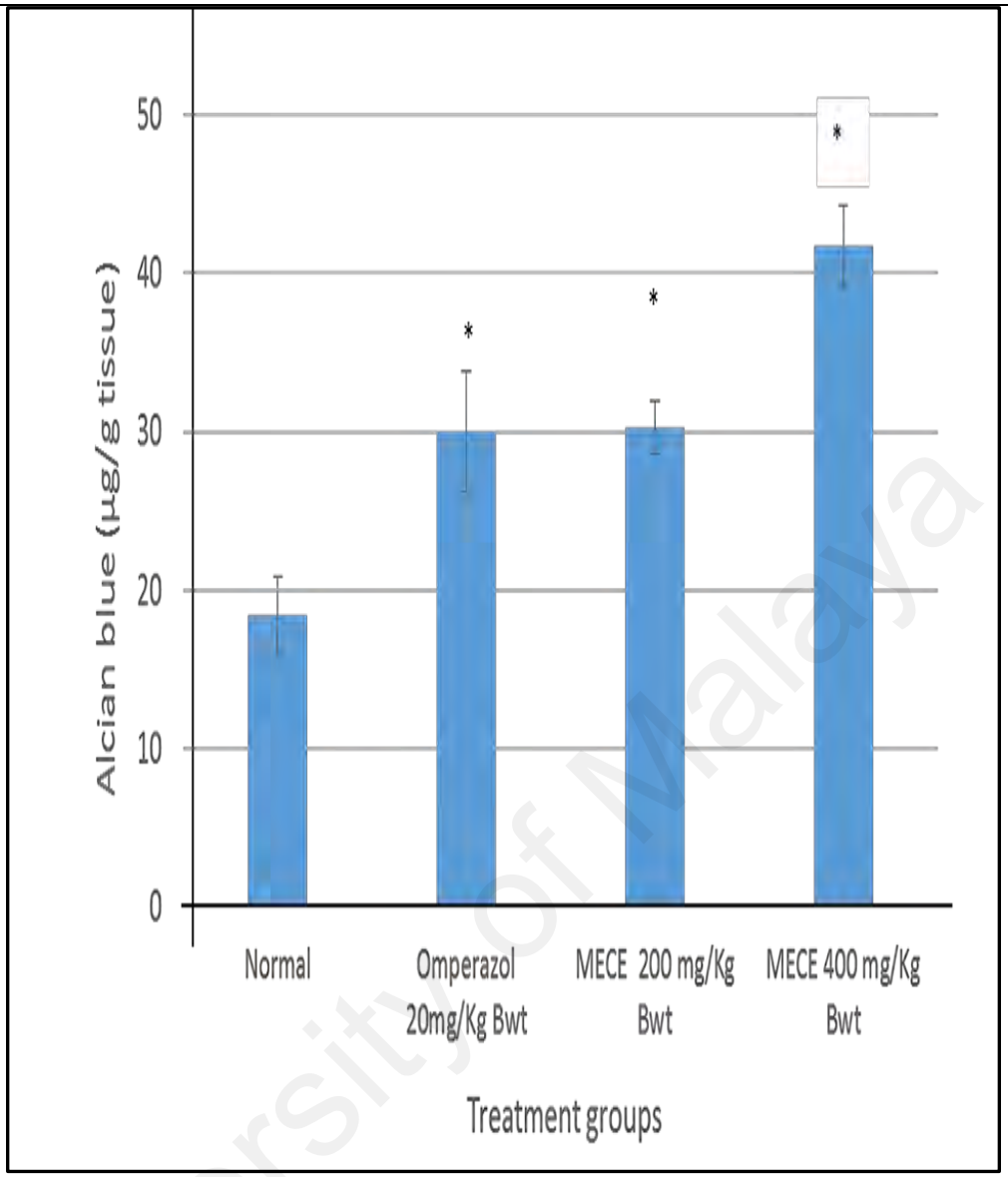

Figure 4.18: Effect of methanolic *C. excavata* extract (MFCE) on rat gastric mucus content in stomach. Values are mean ± S.D. * significant ($P \leq 0.05$) compared with normal control group (6 rats/group).

4.9.2. Rat gastric ulcer model

4.9.2.1. Gastric mucosa

At 200 and 400 mg/kg body weight, MFCE significantly *(p*<0.05) inhibited ulcer formation by 92.6 and 87.8%, respectively (Table 4.14 and Figure. 4.19). There was histological evidence of flattening of gastric mucosa folds and reduction in mucosal damage in these rats upon treatment with the extract and omeprazole.

4.9.2.2. Gastric content, pH, and mucus weight

Treatment with MFCE and omeprazole increased mucus content of the gastric mucosa, with the effect being more pronounced (p<0.05) with MFCE at 400 mg/kg body weight compared with other treatments. The pH of the gastric content of rats treated with MFCE was also significantly (p<0.05) higher than in the untreated rats with ulcer (Table 4.14).

Table 4.14: Effect of methanolic *C. excavata* extract treatment on pH of gastric content, gastric ulcer area and ulcer inhibition in rats

Treatment	pH	Mucus content	Ulcer area (mm^2)	Inhibition (%)
Tween 20 (Normal control)	6.81±0.17	0.92±0.46	0	NA
Ethanol (Untreated control)	3.08±0.11	0.48±0.07	460.8±65.07	NA
Omeprazole (20mg/kg Bwt)	7.14*±0.26	0.82*±0.08	59.40*±23.81	86.3
MFCE (200 mg/kg Bwt)	6.16*±0.08	0.66*±0.18	34.20*±14.46	92.6
MFCE (400 mg/kg Bwt)	6.03*±0.33	1.14*±0.03	56.16*±23.92	87.8

All the values are expressed as the mean ± SEM. *Significant differences at (p<0.05) compared with untreated control. MFCE = methanolic *C. excavata* leaf extract. Bwt = Body weight, NA=not applicable.

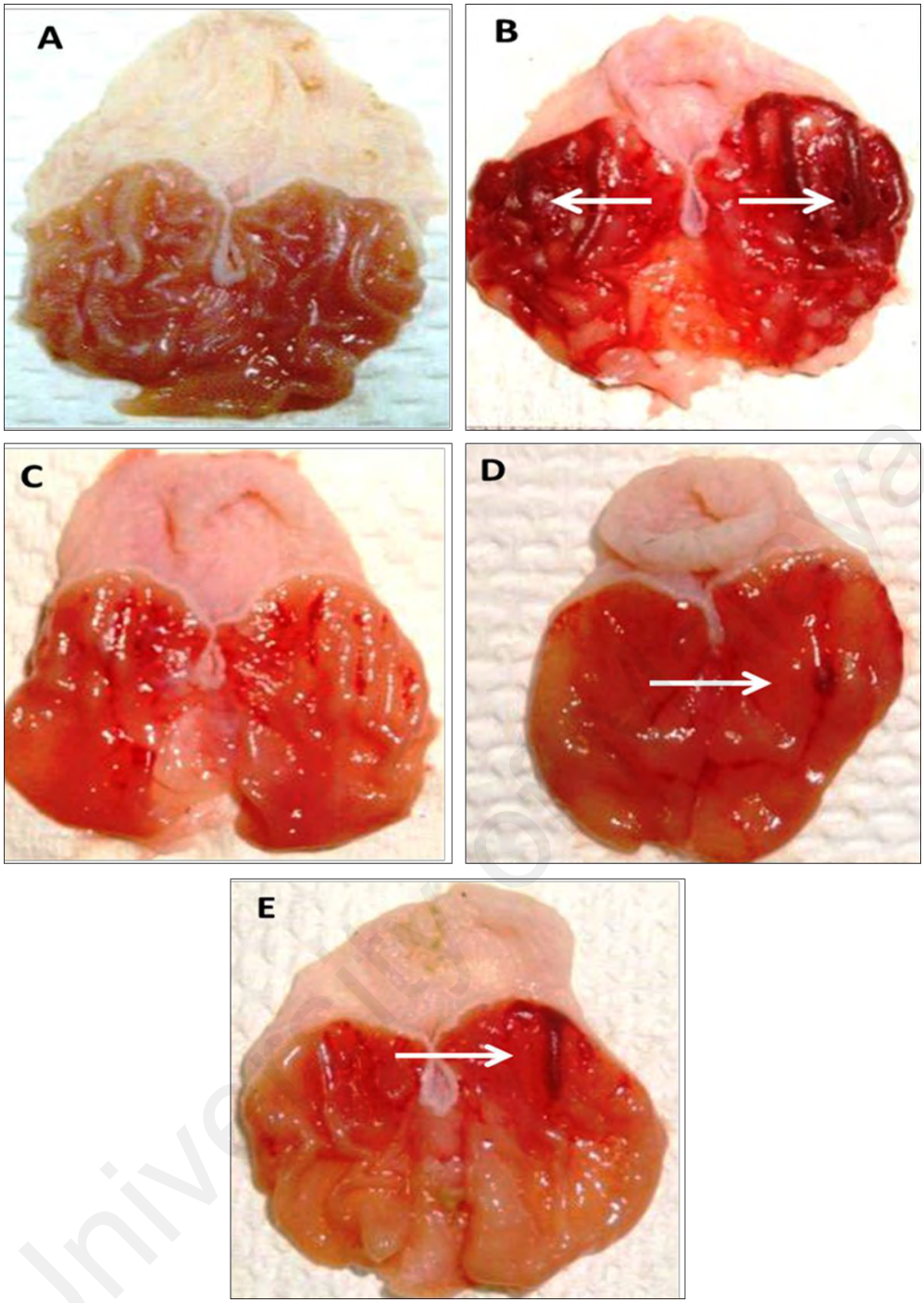

Figure 4.19: Gastric lesions in rats with gastric ulcer treated with omeprazole and methanol *Clausena excavata* extract (MFCE). **(A)** 5% Tween 20%, 5 mL/kg body weight (normal control), **(B)** 5 mL/kg body weight distilled water (ulcer control) showing severe ulceration of the gastric mucosa (arrows), **(C)** 20 mg/kg body weight omeprazole, **(D)** 200 and **(E)** 400 mg/kg body weight MFCE showing mild gastric mucosal ulceration (arrow).

4.9.3. Histological evaluation of gastric mucosa

4.9.3.1. Hematoxylin and eosin (H & E) staining

Hematoxylin and Eosin was performed to assess basic histopathological changes in stomach tissue. Histopathologically, rats with untreated ulcers showed extensive gastric lesions with submucosal oedema and leucocytes infiltration. The ulcer control rats had the highest lesion scores among groups. The stomach of rats pretreated with MFCE showed significantly (p<0.05) lesser ulceration in the gastric mucosa then the untreated control as evident by the smaller ulcer area, lesser or complete absence of oedema and leucocyte infiltration in the submucosal (Figure 4.20 and Table 4.15).

Table 4.15: Gastric tissue lesion score in rat gastric tissue with ulcer treated with methanolic *C. excavata* leaf extract

Treatment	Hemorrhage	Submucosal oedema	Epithelial cell loss	Inflammatory cell Infiltration	Total lesion area (mm^2)
Tween 20 (Normal control)	0	0	0	0	0
Ethanol(Untreated control)	2.8±0.2	2.6±0.3	2.7±0.4	2.3±0.1	10.4
Omeprazole (20mg/kg Bwt)	1.5*±0.4	1.55*±0.2	1.4*±0.1	1.6*±0.3	6.05*
MFCE (200 mg/kg Bwt)	1.56*±0.3	1.41*±0.1	1.49*±0.3	1.32*±0.4	5.78*
MFCE (400 mg/kg Bwt)	0.73*±0.1	0.91*±0.1	0.63*±0.2	0.68*±0.1	2.95*

One unit square is the 2×2 mm area of tissue with lesion. All the values are expressed as the mean ±SEM. Significant differences at *(p<0.05) compared with untreated control. MFCE = Methanolic *C. excavata* extract. Bwt = body weight.

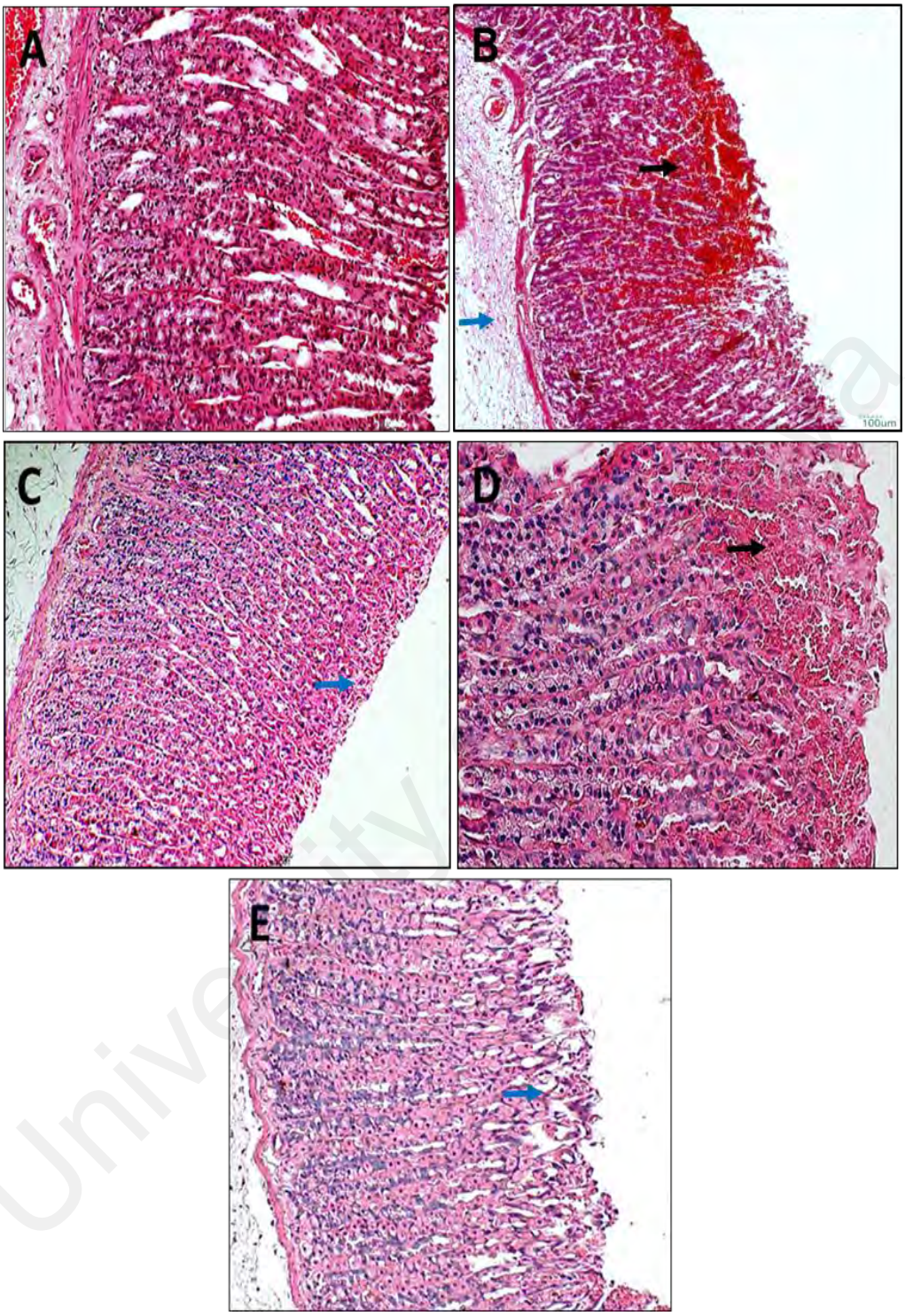

Figure 4.20: Rat gastric tissue with ulcer treated with omeprazole and methanolic *Clausena excavata* extract (MFCE). **(A)** Normal gastric tissue, **(B)** untreated gastric ulcer with extensive haemorrhages (black arrow) and oedema (blue arrow), and treated with **(C)** 20 mg/kg body weight omeprazole, **(D)** 200 **(E)** and 400 mg/kg body weight MFCE. (H & E stain, ×200).

4.9.3.2. Gastric mucus by periodic acid-Schiff (PAS) stain

The histological staining by PAS staining was conducted to detect the level of mucosubstances secreted in stomach, in which glycoprotein react with Schiff's reagent after oxidized to produce magenta color. Under PAS staining, the rats with gastric ulcer treated with MFCE and omeprazole treatments showed significantly (p<0.05) enhanced mucus secretion as seen over the entire lining of gastric mucosa at the surface cells and neck part of the gastric glands. Untreated ulcers did not show mucus secretion on the gastric mucosa (Figure 4.21 and Table 4.16).

Table 4.16: Area of rat gastric mucosal tissue positive for periodic acid-Schiff staining after treatment with methanolic *C. excavata* leaf extract.

Treatment	Stained area (mm^2)
5% Tween 20 (Normal control)	$1.56^{*} \pm 0.2 \times 10^3$
Ethanol (Untreated control)	NA
Omeprazole (20mg/kg Bwt)	$3.19^{*} \pm 0.19 \times 10^3$
MFCE (200 mg/kg Bwt)	$3.02^{*} \pm 0.3 \times 10^3$
MFCE (400 mg/kg Bwt)	$4.67^{*} \pm 0.21 \times 10^3$

All the values are expressed as the mean ±SEM. *Significant differences (P< 0.05) compared with untreated control. MFCE = methanolic extract of *C. excavata* methanolic leaves. Bwt = Body weight, NA=not applicable.

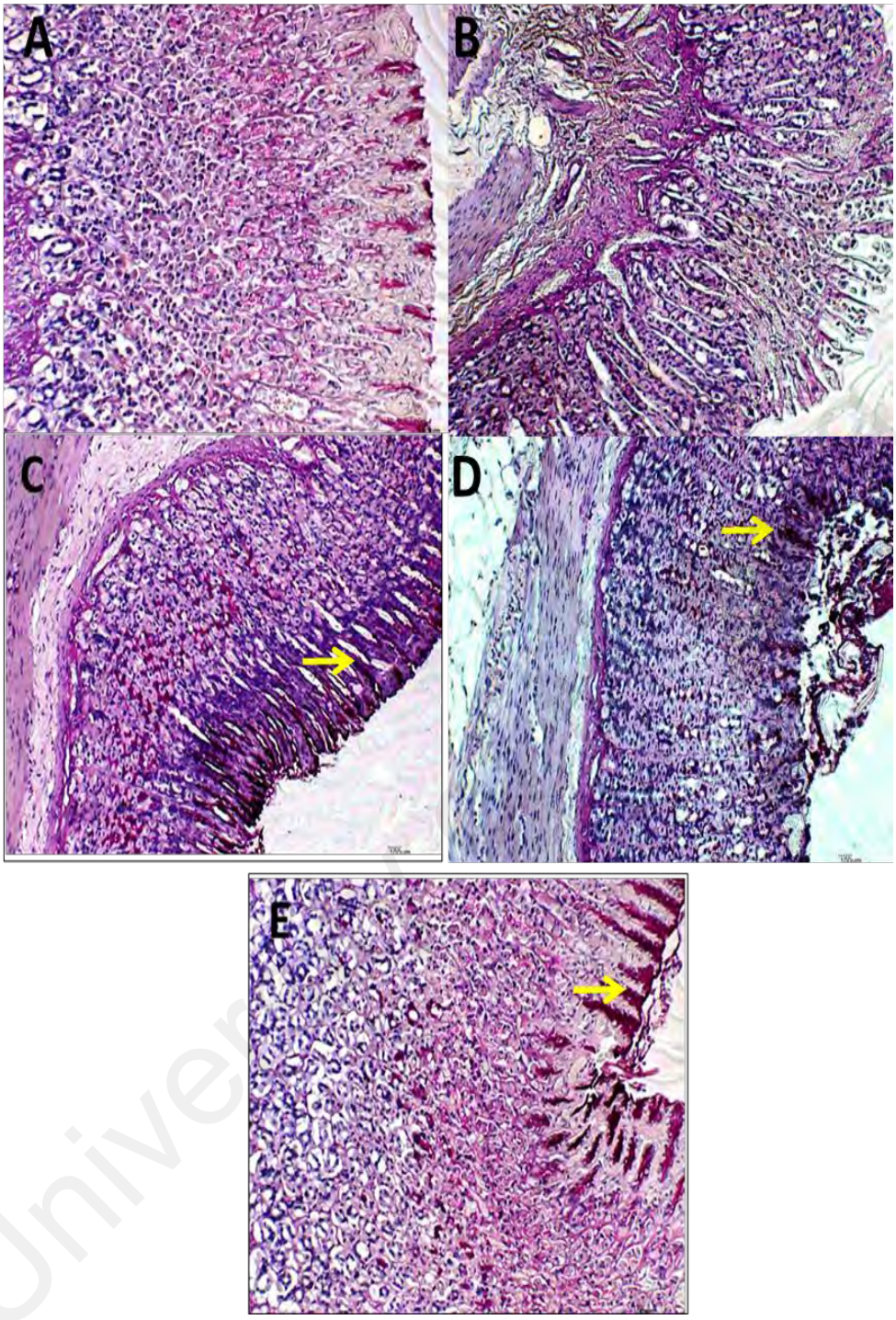

Figure 4.21: Mucus production in rat gastric tissue treated with omeprazole and methanol *Clausena excavate* extract (MFCE). **(A)** Normal gastric tissue (Normal control), **(B)** Untreated ethanol-induced gastric ulcer (negative control), and treatment with **(C)** 20mg/kg body weight omeprazole (positive control), **(D)** 200 and **(E)** 400 mg/kg body weight MFCE. Magenta coloration (arrow) in the epithelial cells indicates increased gastric gland secretion. The most intense color is in gastric tissue treated with 400 mg/kg body weight MFCE. (Periodic acid-Schiff stain, ×200).

4.9.3.3. Immunohistochemistry analysis of HSP70, BAX and TGF- β

Immunohistochemistry was performed to show the expression of HSP70, BAX and TGF- β proteins in stomach. Since HSP70 plays an important role in protection the cells against stresses and preventing damage, while the BAX is a proliferative suppressor, which promotes apoptosis, TGF-β which is reported to be unique in its effect on cell growth and differentiation. Therefore, we selected these proteins in present study. Unlike in untreated gastric mucosa with ulcer that showed up-regulation of BAX protein, in the MFCE-treated mucosa the expression of this protein was down-regulated (Figure 4.22 and Table 4.17). On the other hand, the HSP70 expression was up-regulated in both omeprazole and MFCE-treated groups. The effect of MFCE on HSP70 expression was dose-dependent, with greater expression at the higher dose of the extract (Figure 4.23 and Table 4.18). TGF β was upregulated in the MFCE-treatment group but this was not statistically significant among MFCE-treated groups (Figure 4.24 and Table 4.19).

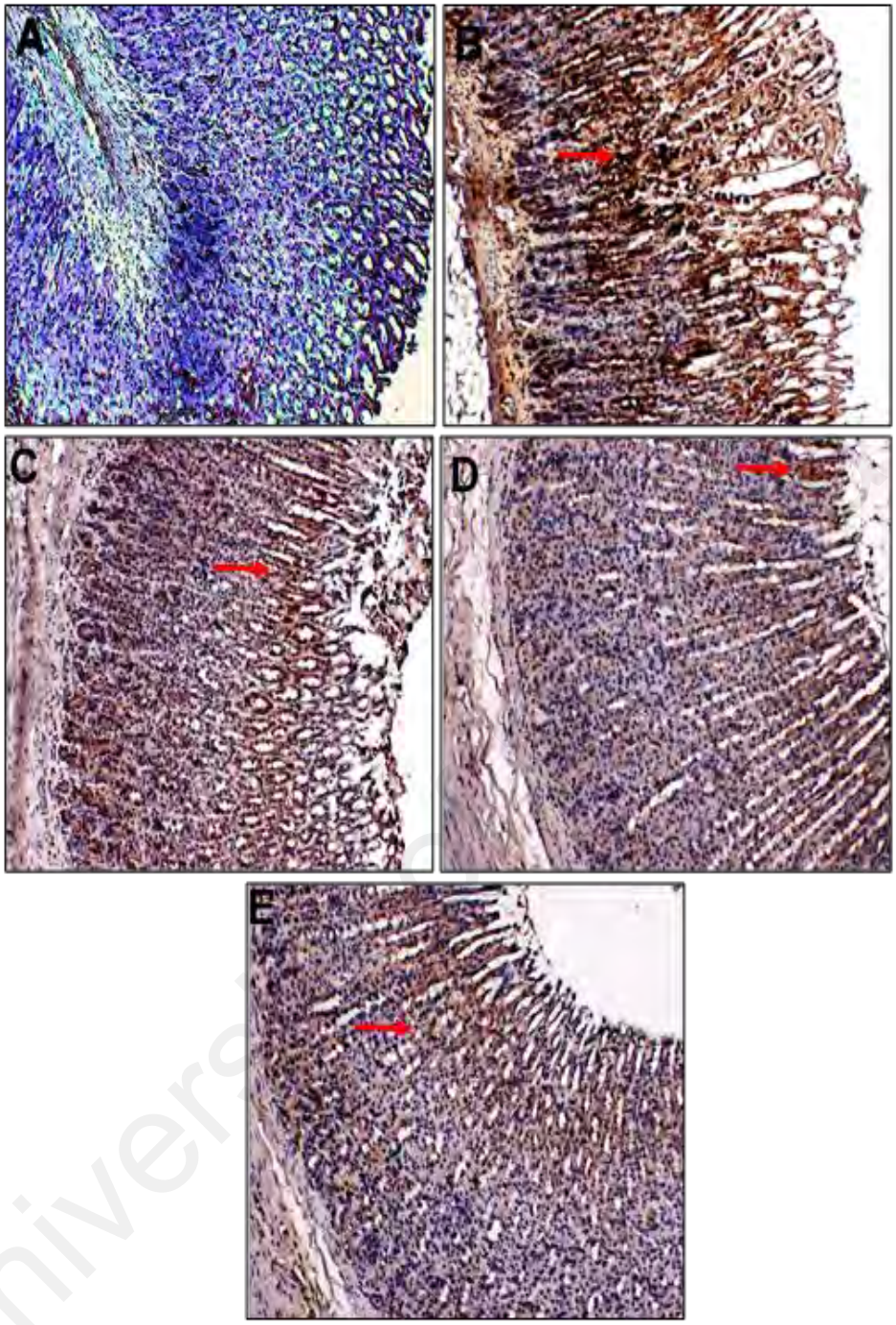

Figure 4.22: BAX protein expression in rat gastric tissue with ulcer treated with omeprazole and methanol *Clausena excavata* extract (MFCE). **(A)** Normal control, **(B)** untreated ethanol-induced ulcer showing over-expression of BAX proteins in the epithelial cells, treatment with **(C)** 20 mg/kg body weight omeprazole **(D)** 200 and **(E)** 400 mg/kg body methanolic *C. excavate* extract showing reduced BAX protein expression in the apical epithelial cells. Positive staining shown by the dark brown coulor. (Immunoperoxidase stain, ×200).

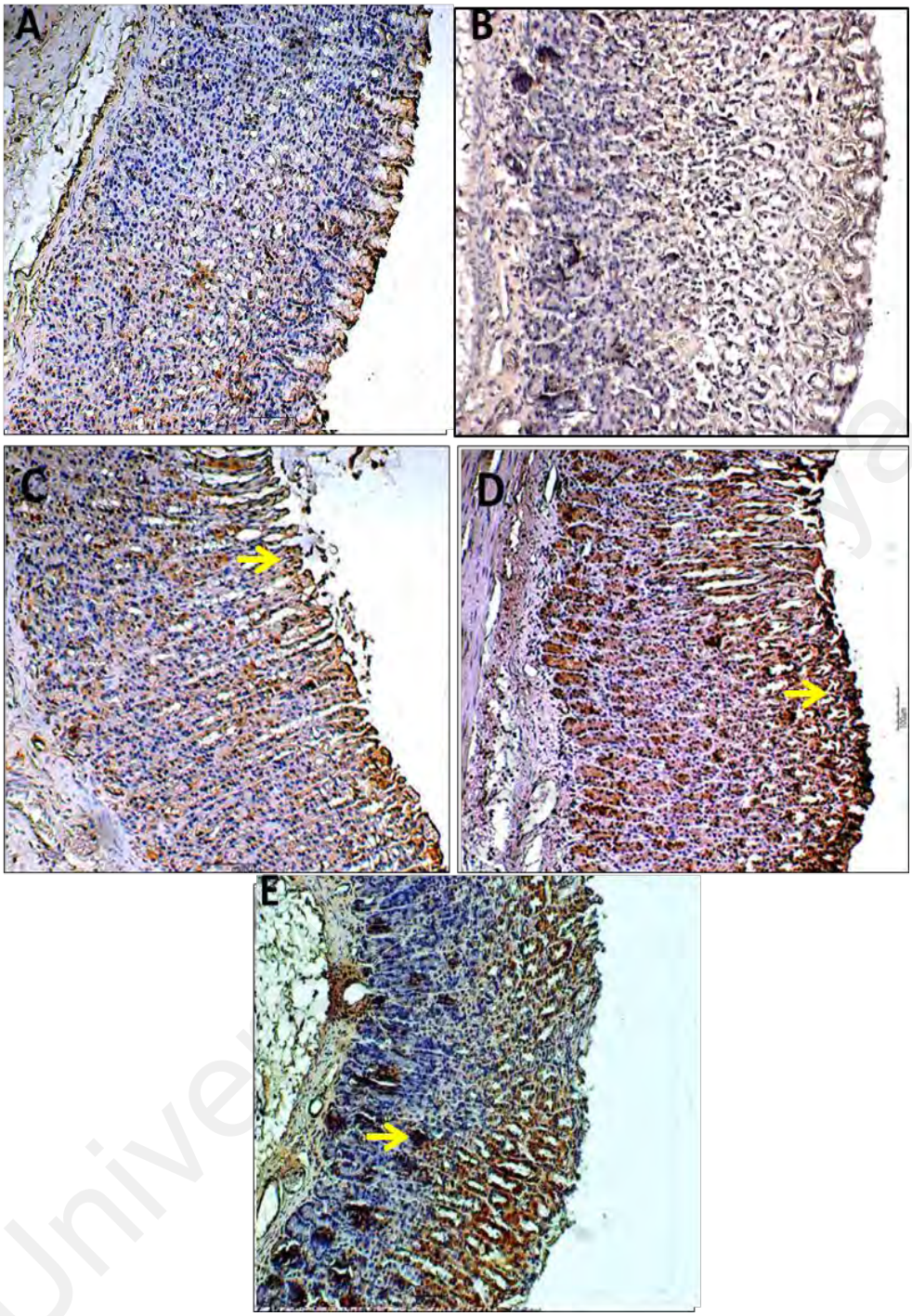

Figure 4.23: HSP70 expression in rat gastric tissue with ulcer treated with omeprazole and methanol *Clausena excavate* extract (MFCE). **(A)** Normal control, **(B)** untreated ethanol-induced ulcer showing under-expression of HSP70 proteins in the epithelial cells, treatment with **(C)** 20 mg/kg body weight omeprazole **(D)** 200 and **(E)** 400 mg/kg body MFCE. Increased HSP70 expression was seen after treatment with 400 mg/kg body weight extract. Arrows show positive expression. (Immunoperoxidase stain, ×200).

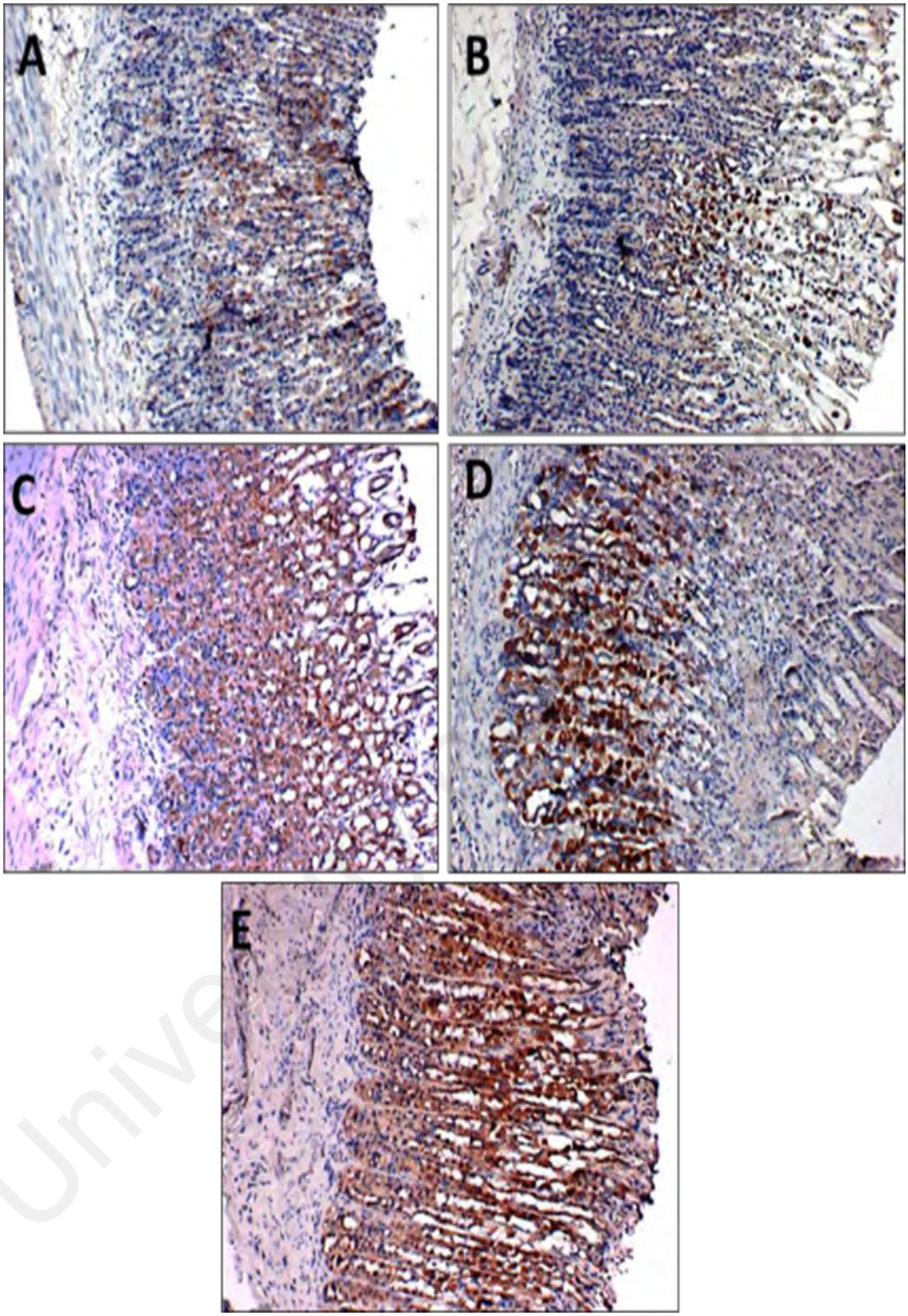

Figure 4.24: TGF-β protein expression in rat gastric tissue with ulcer treated with omeprazole and methanol *Clausena excavata* extract (MFCE). **(A)** Normal control, **(B)** untreated ethanol-induced ulcer showing under-expression of TGF-β proteins in the epithelial cells, treatment with **(C)** 20 mg/kg body weight omeprazole **(D**) 200 and **(E)** 400 mg/kg body MFCE showing increased TGF-β protein expression in the apical epithelial cells. Arrow show positive expression. (Immunoperoxidase stain, ×200).

Table 4.17: Area of rat gastric mucosal tissue positive for BAX protein immunoperoxidase staining after treatment with methanolic *C. excavata* extract.

Treatment	Stained area ($\times 10^3$) (μm^2)	% Area
5% Tween 20 v/v (#Normal control)	0.00	0
Ethanol (5mL/kg Bwt) (Untreated control)	48.0 ±0.4	40
Omeprazole (20mg/kg Bwt)	21.2* ±2.6	17
MFCE (200 mg/kg Bwt)	15.1* ±2.1	12
MFCE (400 mg/kg Bwt)	4.2* ±1.3	4

All the values are expressed as the mean ± SEM. * Significant differences at (p<0.05) compared with ulcer control. #Treated with 5% Tween 20 only. MFCE = methanol *C. excavata* extract. Bwt = Body weight.

Table 4.18: Area of rat gastric mucosal tissue positive for HSP 70 protein immunoperoxidase staining after treatment with methanolic *C. excavata* extract.

Treatment	Stained area ($\times 10^3$) (μm^2)	% Area
5% Tween 20 v/v (#Normal control)	5.4±2.2	4
Ethanol (5mL/kg Bwt) (Untreated control)	6.3±3.1	5
Omeprazole (20mg/kg Bwt)	32.4*±2.9	22
MFCE (200 mg/kg Bwt)	36.4*±3.3	25
MFCE0 (40 mg/kg Bwt)	46.3*±3.7	33

All the values are expressed as the mean ± SEM. * Significant differences at (p < 0.05) compared with ulcer control. #Treated with 5% Tween 20 only. MFCE = methanol *C. excavata* extract. Bwt = Body weight.

Table 4.19: Area of rat gastric mucosal tissue positive for TGF-β protein immunoperoxidase staining after treatment with methanolic *C. excavata* extract.

Treatment	Stained area ($\times 10^3$) (μm^2)	% Area
5% Tween 20 v/v (#Normal control)	3.6±0.8	3
Ethanol (5mL/kg Bwt) (Untreated control)	4.5±0.1	4
Omeprazole (20mg/kg Bwt)	44.2*±0.9	34
MFCE (200 mg/kg Bwt)	31.6*±2.3	20
MFCE (400 mg/kg Bwt)	35.1*±3.4	24

All the values are expressed as the mean ± SEM. * Significant differences at (p < 0.05) compared with ulcer control. #Treated with 5% Tween 20 only. MFCE = methanol *C. excavata* leaf extract. Bwt = Body weight.

4.9.4. Effect of *C. excavata* on Prostaglandin E2 and Antioxidant activity

Since prostaglandins control gastric acid secretion and enhance gastric mucus productions, thus playing a significant protective role by conferring the gastric mucosa ability to resist injury caused by noxious compounds. Reactive oxidative radicals are scavenged by the antioxidant defense system of the body and accumulation of ROS increases LPO, so measurement of these biomarker are important in development gastric mucosal lesions.

The SOD, CAT, GPx activities and PGE2 concentrations were significantly (p<0.05) higher in the gastric tissue with ulcer treated with MFCE and omeprazole than the untreated tissue (Figures 4.25 a, b, c, d & e). In contrast, the extract produced significantly (p< 0.05) lower lipid peroxidation activity in all treated than untreated gastric tissue with ulcer. The results show that the antioxidant activities in gastric tissue treated with MFCE are greater than in tissues treated with omeprazole.

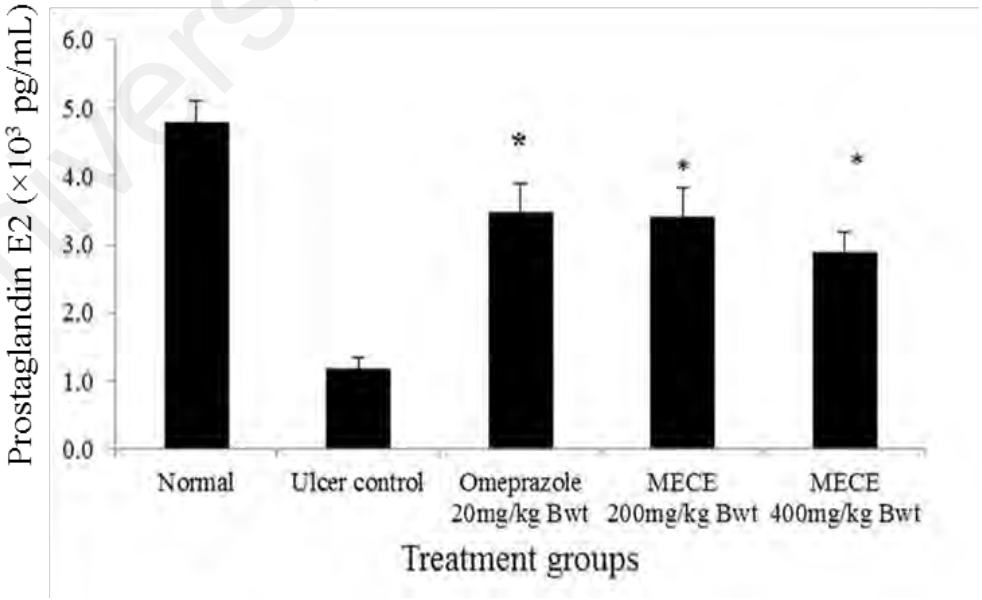

Figure 4.25 a: Effect of omeprazole and methanol *Clausena excavata* extract (MFCE) on prostaglandin E2 (PGE2) level in rat gastric ulcer. All data are mean ± SD of 6 rats per group. The PGE2 concentrations are higher in omeprazole and MFCE treated than untreated ulcers. *Significant at *P<0.05*.

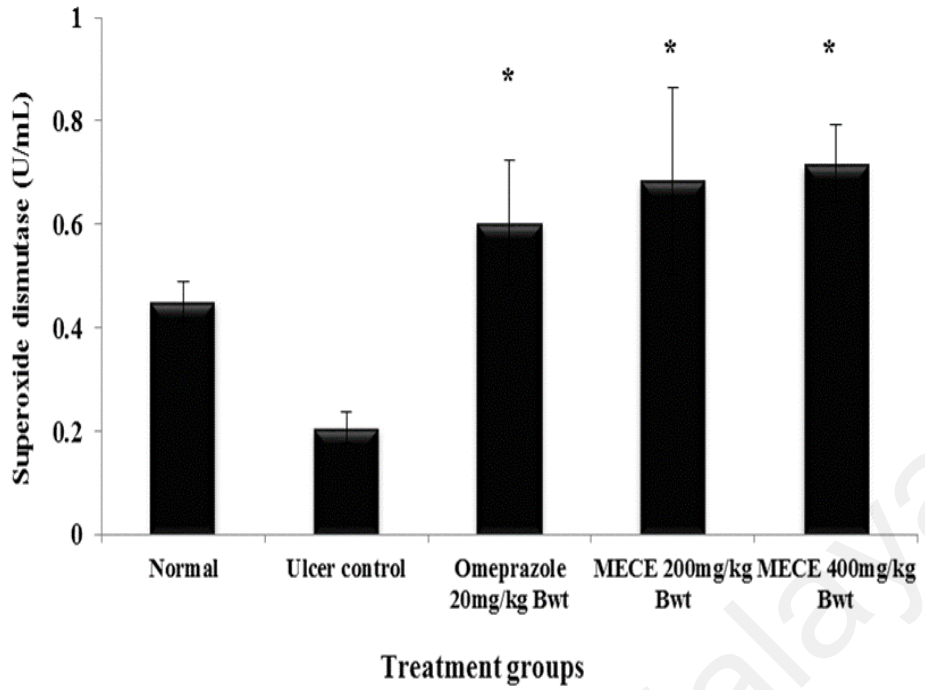

Figure 4.25 b: Effect of omeprazole and methanol *Clausena excavata* extract (MFCE) on superoxide dismutase (SOD) level in rat gastric ulcer. The SOD levels were significantly higher in omeprazole and MFCE treated than untreated ulcers. Values are presented as mean ± SD of 6 rats/group. *Significant at (P< 0.05).

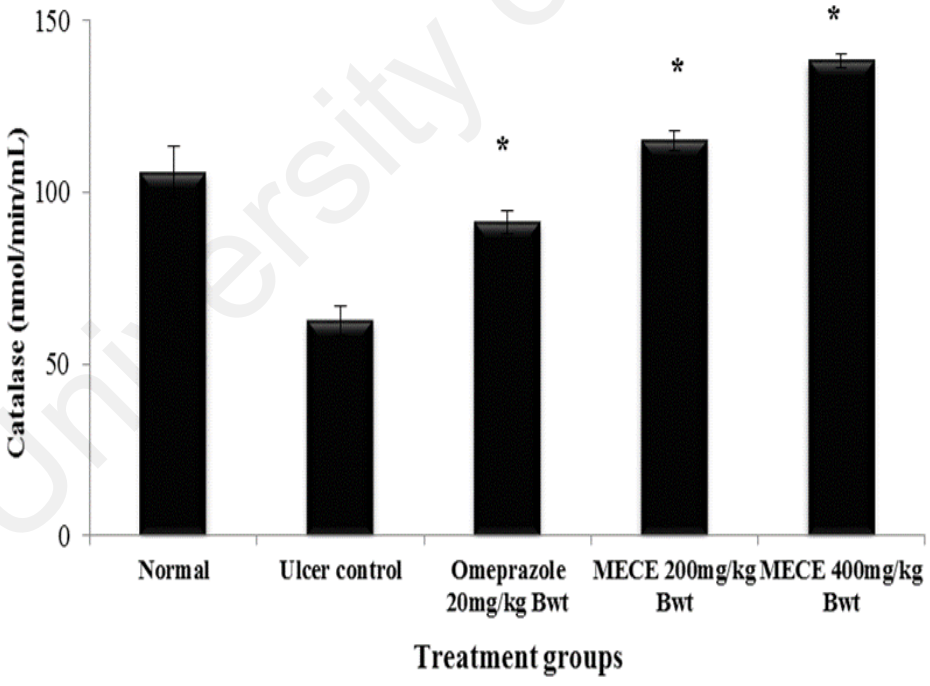

Figure 4.25 c: Effect of omeprazole and methanol *Clausena excavata* extract (MFCE) on catalase (CAT) level in rat gastric ulcer. The CAT level were higher in omeprazole and MFCE treated than untreated ulcer. Values are presented as mean ± SD of 6 rats/group.*significant at $P < 0.05$.

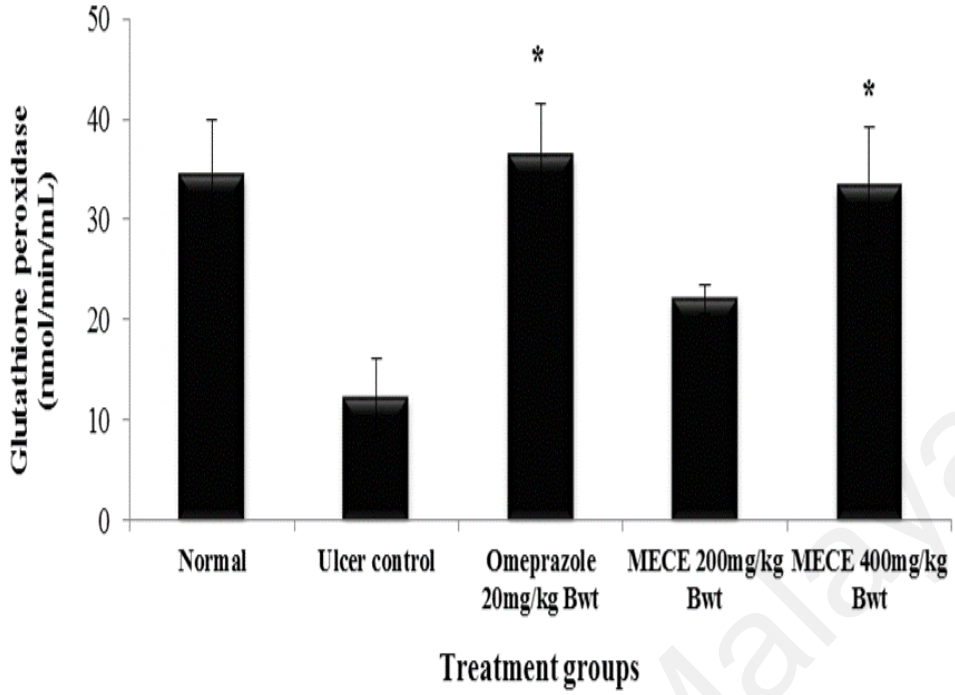

Figure 4.25 d: Effect of omeprazole and methanol *Clausena excavata* extract (MFCE) on glutathione peroxidase (GPx) in rat gastric ulcer. The GPx level was higher in omeprazole and 400 mg/kg body weight MFCE-treated than untreated gastric ulcer. Values are presented as mean ± SD of 6 rats/group. *significant at $P < 0.05$.

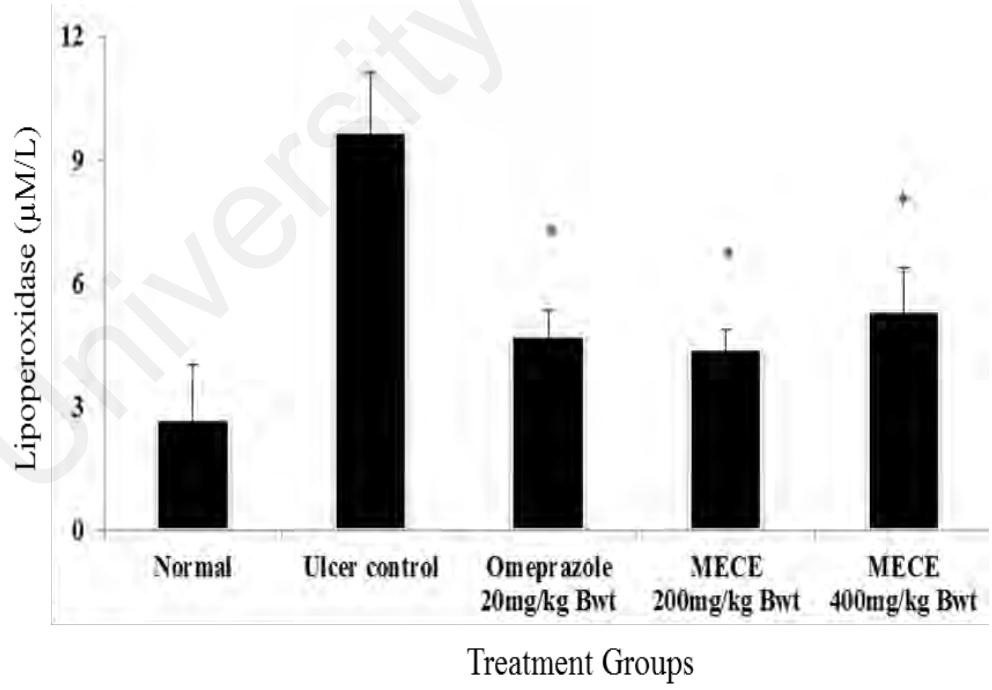

Figure 4.25 e: Effect of omeprazole and methanol *Clausena excavata* extract (MFCE) on lipid peroxidation (LPO) in rat gastric ulcer. The LPO level are significantly lower in omeprazole and MFCE-treated than untreated gastric ulcer. Values are presented as mean ± SD of 6 rats/group. *Significant at $P < 0.05$.

4.9.5. Estimation of TNF-α -6 and IL-10

Since ethanol ingestion causes releasing the proinflammatory cytokines as TNF-α and IL-6, which are associated with increased gastric tissue damage, therefore in this study we investigated the effect of MFCE on these cytokines. The IL-10 levels were significantly (P <0.05) higher in rat gastric ulcer pretreated with MFCE than untreated control ulcer. By contrast, the levels of TNF-α and IL-6 were significantly (P<0.05) lower in these treated groups compared to the untreated control ulcer (Figure 4.26).

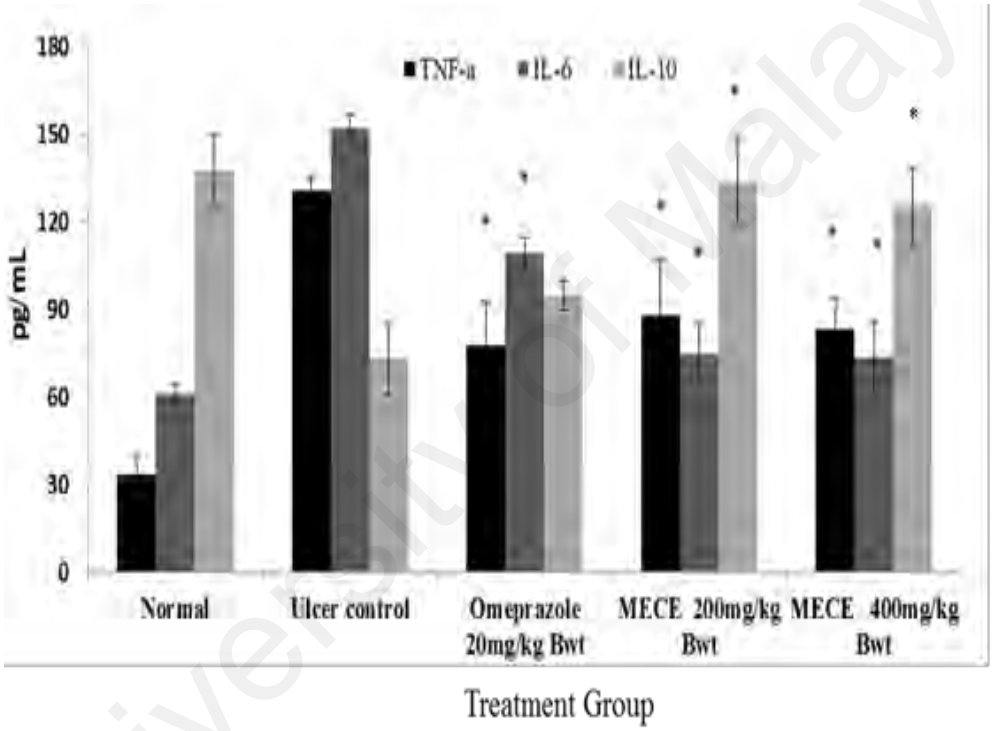

Figure 4.26: Effect of omeprazole and methanol *Clausena excavata* extract (MFCE) on tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and -10 (IL-10). The TNF-α and IL-6 are significantly lower and IL-10 higher in the omeprazole and MFCE-treated than the untreated control ulcers. Values are expressed as mean ± S.D of 6 rats/group. *Significant at $p < 0.05$.

CHAPTER 5: DISCUSSION

5.1. Evaluation of biological activities of *C. excavata* solvent extracts *in vitro*

Over the past years, a handful of pharmacologically active plant constituents belonging to the family Rutaceae has been identified, these include methoxyflavones, furoquinoline alkaloids, furanone-coumarins, and flavonoids. These constituents were shown to have remarkable effects on inflammation, viral infections, and digestive system disorders such as gastritis and enteritis (He et al., 2000; Xin et al., 2008; Nakamura et al., 2009; Guntupalli et al., 2013; Venugopala et al., 2013). *Clausena excavata*, in particular, possesses a wide range of pharmacological properties which range from the antioxidant properties (Guntupalli et al., 2012), hyperglycemic, anti-rhinitis, anti-inflammatory (Sakong et al., 2011), anti-nociceptive (Rahman et al., 2002), anti-viral, anti-cancer and anti-fungal activities (Su et al., 2009; Kumar et al., 2012).

The antioxidant activity of plants has become important in recent years because of its effectiveness in general health by playing an important role in the protection of the body from the adverse effects of over production of reactive oxygen species (Gupta et al., 2005). In most studies, antioxidant activities of *C. excavata* used the bark and stem of this plant, but not the leaves. In the present study, the extraction of the leaves with solvents such as methanol, ethyl acetate, chloroform and petroleum ether fractions, showed variations in the potency of antioxidant activities as measured by FRAP and DPPH, which showed that difference in extraction solvents affected the antioxidant properties of the extract. Polar solvents such as water, methanol, ethanol and ethyl acetate were more suitable as extraction solvents in recovering polyphenols from the matrix of plants (Khan et al., 2012). Another study showed that the type of solvent significantly influenced the level of antioxidants in plant extracts (Lapornik et al., 2005).

In the present study, the effective antioxidant ability of *C. excavata* was demonstrated through the ferric reducing ability and radical scavenging action. FRAP assay is considered to be an accurate and simple method for evaluating the antioxidant capacity of therapeutic compounds. The reducing ability of any compound has been established to depend on its ability to donate H^+ electron (Al-Abd et al., 2015). In DPPH radical scavenging assay, unstable free radical DPPH receives a hydrogen atom from antioxidant compound so that it will be converted into a stable form (Kedare & singh, 2011). Among these four fractions, the methanolic fraction was observed to strongly reduce $FeCl_3$ and showed a DHPP scavenging activity with IC_{50} near to quercetin. This finding corroborates with other studies which reported that the methanolic extract possesses higher antioxidant activity which is linked to its high polarity index when compared to other plant extracts in various model systems (Iloki-Assanga et al., 2015). This observation suggests that the methanolic fraction of *C. excavata* is be able to donate electrons to free radicals due to its phenolic and flavonoid contents (Moure et al., 2000; Jayaprakasha et al., 2008). It is well-documented that phenolic compounds are among the principal and effective antioxidant constituents in plant foods, and its antioxidant activity resulting from their ability to scavenge free radicals through their hydroxyl groups (Velioglu et al., 1998; Altaf et al., 2013). Previous *in vitro* studies have reported that *C.excavata* leaves with high phenolic content inhibited lipid peroxidation (Sakong et al., 2011; Guntupalli et al., 2012). In the present study the MFCE leaves had the highest amount of phenolic compounds, so the high antioxidant activity of the methanolic fraction could be due to its ability to scavenge free radicals. Of all the polyphenolic compounds, flavonoids are said to be the major scavengers of superoxide anions, peroxyl radical and other oxidizing species by virtue of their wide spectrum of medicinal actions (Iloki-Assanga et al., 2015). In this study, the chloroform extract of *C. excavata* showed the highest concentration of flavonoids, which was comparable with

concentrations reported by other researchers (Hossain et al., 2011; Ekeanyanwu & Njoky, 2014). Thus, it is not surprising that *C. excavata*, with its potent phenolic and antioxidant properties can be used to treat of various oxidative stress-related diseases.

Since the biological activity of extracts have a strong relationship with the solvents employed on the basis of their polarities, different solvent extracts from *C. excavata* leaves were used to determine their biocompatibility on *in vitro* cell lines including; keratinocytes, fibroblast and macrophages. The MOH extract of *C. excavata* used in this study had a cell viability of 71% and 47% at 400 µg/mL in HaCaT and Vero cells, respectively. It was also observed that at low doses, the MOH and PT extracts of *C. excavata* enhanced the proliferation of these cells. The MFCE contains quercetin and myricetin, which have been shown to enhance the growth of the normal human skin fibroblast (FS5) cell line by conferring protection on against hydrogen peroxide-induced damage (Adetutu, 2009). However, the EA and CH extracts showed a significant reduction in the percentage of cell viability in HaCaT cells with 19% and 27% viability at 400 µg/mL, respectively. This may indicate that non-polar active components are responsible for the antiproliferative activity of this plant as previously reported in other plants (Talib & Mahasneh, 2010). The percentage of cell viability observed in Vero cells was similar to that of HaCaT cells, but Vero cells were more resistant (51% and 78% at 400 µg/mL) than HaCaT cells following exposure to EA and CH. This selectivity could be due to the sensitivity of the cell line to the active compounds in the extract (Ayob et al., 2014). However, the EA and CH fractions showed a significant reduction in the percentage of cell viability in macrophage cells with 17% and 20% viability at 400 µg/mL, respectively, the methanolic fraction did not show any toxicity towards the macrophage cell line even at a high dose. Since macrophages produce macrophage colony stimulating factor (M-CSF), which play a

significant role in healing of gastric ulcers through promotion of angiogenesis, treatment of gastric ulcers with MFCE should not inhibit healing (Kawahara et al., 2011).

In wound healing, bacterial infections play an important role in the healing by release of free radicals and lytic enzymes at the wound site. These enzymes have the ability to destroy the lipids, proteins and extracellular matrix that delay the onset of the proliferative and remodeling phases of wound healing process (Udegbunam et al., 2014). The pathogens tested in this study namely; *S. aureus, B.subtilis, A. anitratus* and *P. aeruginosa* are frequently incriminated in wounds infections (Allen & Green, 1987; Abrahamian & Goldstein, 2011). Previous studies have reported that plants with antibacterial and antioxidant properties accelerate wound healing by increasing the rate of wound contraction and re-epithelialization (Nayak & pereira, 2006; Udegbunam et al., 2014). Another study reported that antimicrobial property attributed to the presence of marker compounds like: phenolic/flavonoids-based compounds and could be a good therapeutic agent for accelerating wound healing process (Houghton et al., 2005; Geethalakshmi et al., 2013; Udegbunam et al., 2014). In the present study, fractions of *C. excavata* demonstrated antibacterial activity through inhibition of growth of these organisms suggesting this plant may facilitate wound healing through antibacterial action. It has been reported earlier that gram-negative bacteria are usually more resistant to the plant-origin antimicrobials and even show no effect compared to Gram-positive bacteria (Biswas et al., 2013). Another reports have shown that several compounds isolated from the roots of *C. excavata* exhibited profound antimicrobial properties (Wu & Furukawa, 1982).

To investigate the healing properties of MFCE, which is attributable to its antioxidant and antimicrobial activities, which in turn result from high phenolic content, we perfoemed classical scratch assay.

The scratch wound test assay adopted fibroblast proliferation and migration as indicators to assess the efficacy of active methanolic extract *in vitro*, because fibroblast is known to play an essential role in wound healing and closure (Basso et al., 2012). Since scarring of the skin and other body parts are due to excessive proliferation of fibroblast cells and its ECM, fibroblast cells are major targets in therapeutic drug design (Singh et al, 2014). Previous research on wound healing have shown the efficacy of this assay in wound-healing *in vitro* models (Guler et al., 2014). Enhancement of fibroblast proliferation and migration by MFCE serve as an indicator for enhancement of wound healing by this fraction. This finding is similar to what was reported in an earlier study where stimulation of fibroblast proliferation and migration was used as an indicator for regulation of angiogenesis, inflammation and tissue regeneration (Tam et al., 2011 Guler et al., 2014).

Based on the *in vitro* screening results, methanol extract of *C. excavata* had high antioxidant and total phenolic contents, optimal proliferation of HaCaT and macrophage cell lines, fibroblast migration and antibacterial activity, therefore the methanolic fraction of the leaves of *C. excavata* was subsequently selected for phytochemical screening, and animal studies.

Initial investigation using LCMS/MS revealed by the chromatograms of the methanolic fraction indicate that *C. excavata* leaves are rich in phenolic/flavonoids-based compounds (Ito et al., 2000). Notably, the compounds in the methanolic fraction of the leaves possess several biological activities such as coumarin, stimulator of gastric prostaglandin and mucus production (Moure et al., 2000; Nakamura et al., 2009; Mogana, et al., 2013; Venugopala, et al., 2013), quercetin glycoside, myricetin glucosides and kaempferol, which belong to a group of flavonoid compounds have been shown to have high anti-inflammatory and antioxidant activities as well as prevention of gastric ulcer and enhancement of wound healing *in vitro* via scratch-wound healing

assays on HaCaT keratinocytes (Calderon-Montano et al., 2011; Clericuzio et al., 2012; Krishnappa et al., 2014).

5.2. Acute toxicity assessment of methanolic extract

Natural products such as plant extracts have been used in therapeutic management of several diseases. Unfortunately, many of these compounds were known to be toxic to life-forms (Parra et al., 2001). Among these compounds, some may exert the toxicity once taken, while others are toxic when give over an extended period. Given these reasons, evaluating the acute toxicity of medicinal compounds for human use is necessary before using them in the proposed therapy (Ateba et al., 2014). The present study showed that during 14 days of the experimental period, there was no mortality or symptoms of toxicity in all the rats at all doses (2000 and 5000 mg/kg). The LD50 of MFCE leaves in both sexes of rats was greater than 5000 mg/kg body weight. This is in accordance with an earlier study by Puongtip et al. (2011), which reported that oral LD50 of the methanolic extract from the stem of *C. excavata* is more than 5000 mg/kg body weight in both sexes in rats. Therefore, the high LD50 of MFCE leaves indicates that the extract is relatively safe, especially for oral administration. According to Ecobichon, (1997) and OECD 2001, any test compound that does not produce death at a dose exceeding 5 g/kg body weight is considered nontoxic.

Change in body weight and relative organ weight of animals is an indicator of adverse effect of administered drugs or chemicals (Teo et al., 2002; Mohamed et al., 2011). The organs of these animals tend to swell or become damaged, which will subsequently alter their organ-to-body weight ratios upon exposure to toxic substances. The hematopoietic system is considered as one of the most sensitive system affected by toxic compounds. Ye et al. (2005) conducted an acute toxicity analysis, where they evaluated the biochemical functions of the renal and liver systems of both control and

compound treated rats (Ekeanyanwu & Njoky, 2014). In this study, the body weights of rats (male and female) treated with MFCE leaves increased gradually but insignificantly ($p>0.05$) compared with the control rats. Similarly, the relative organ weights showed no significant ($p>0.05$) difference between test and control groups. Hematological analysis showed that there are no significant differences between all of the treated groups as compared to the control groups. The level of RBC and HGB, did not change, indicating that polycythemia and hemolytic anemia were not induced. On the other hand, increased serum creatinine is a good indicator for assessment of glomerular filtration and abnormal kidney function while simultaneous increases in serum AST and ALT, the liver enzymes associated with hepatopathy (Rhiouani et al., 2008). Our study showed significant ($p<0.05$) but variable increases in serum enzymes according to dose and sex. High dose MFCE treatment seemed to affect the males slightly more than the females. Serum bilirubin concentration was significantly ($p<0.05$) elevated in treated female rats only. In this case, increase in ALP level is associated with increased hepatic synthesis, in the presence of increasing biliary pressure and obstruction in the normal bile flow as a result of damage or inflammation (Ekeanyanwu & Njoky, 2014). Significant increase in BUN without abnormality in serum creatinine concentration in female rats treated with a high dose of MFCE leaves is indicative of hem concentration and/or high dietary protein rather than kidney impairment (Ashafa et al., 2009). This assertion is based on the fact that serum creatinine is more accurate marker of glomerular filtration and kidney function than urea. The mild to moderate histopathological changes in the kidneys and liver, coupled with relatively lack of change in hematological and other serum biochemical parameters further confirms that MFCE is nontoxic and safe to be used as a therapeutic extract at low to moderate doses.

5.3. Wound healing evaluation parameters

Cutaneous wound healing is reportedly a complex process that involves a series of immune-mediated cellular and molecular interactions which begins with wound contraction, which is part of normal healing process that closes wounds to the external environment. Thereafter, the proliferative phase sets in, which is characterized by tissue granulation formation, and finally epithelialization developing and remodeling of damaged tissue (Roy et al., 2009; Hsu & Mustoe, 2010). In recent years, ethno pharmacology has become a focus area for researchers in the search for natural materials with potent antioxidant and wound healing properties (Geethalakshmiet et al., 2013). The reactive oxygen species (ROS) generation by inflammatory cells within the dermis plays an integral role in mediating wound healing by affecting proliferation and cell survival signaling to alter apoptotic pathways in the skin and this contributes significantly to pathogenesis of impaired skin wound healing (Lee et al., 2012). Since wound healing can be accelerated by enhanced radical scavenging power (Abdulla et al., 2011), *C. excavata* extract containing coumarins, flavonoids with a wide array of biological properties and demonstrated effective antioxidant ability through various mechanisms of ferric reducing ability and radical scavenging action, was investigated for its wound healing properties through its antioxidant capacity.

The choice of topical application of MFCE was made because of the advantages associated with such formulation; availability of high and sustained concentration of these agents at the site of infection with limited total amount needed and limited potential for systemic absorption and toxicity (Lipsky & Hoey, 2009). Similarly, it has been reported in a study that, topically applied drugs are known to be effective in faster wound closure and overall healing due to larger availability of the drug at the desired wound site (Ponrasu et al., 2014). The difficulty of applying and maintaining the topical

formulation of MFCE and the duration of treatment while maintaining absorption and penetration of the formulation to the wounded skin area are part of the challenges and limitations of this study.

The wound healing properties of MFCE was determined in inflicted wounds in a rat model. During treatment, the MFCE did not cause irritation or pain to the animals as evident by the lack of discomfort in the rats. The main finding of this study is that the topical application of the bioactive fraction of *C. excavata* increased wound contraction in all the treated groups by days 10 and 15 compared to the untreated group. The observed healing impairment in the control group could be accorded to the high level of reactive oxygen species due to higher leukocytes infiltration, which in turn impairs keratinocyte, endothelial cell, fibroblast, and collagen metabolism. Similarly, the observed accelerative wound healing upon *C. excavata* treatment could be attributed to the radical scavenged activity of the *C. excavata*.

Transforming growth factor beta type appreciative1 (TGF-β1) plays an important role in wound healing as a regulators of the deposition of extracellular matrix (ECM), it enhances the gene transcription of collagen, inhibits the production of collagenase in charge of matrix breakdown, stimulates the metalloprotease inhibitor and collagen formation *in vivo* (Upadhyay et al., 2013; Diegelmann & Evans, 2004). The study also showed that MFCE treatment resulted in increased TGF-β expression in the skin wound. Among the effects of TGF-β is inhibiting lymphocyte infiltration (Eisenstein & Williams 2009; Kubiczkova et al., 2012). What this does is to facilitate wound repair while preventing development of immune response in the treated tissues. This observation is supported by the histopathological study. The skin treated with MFCE showed decreased inflammatory response, degeneration, granulation tissue, angiogenesis rate, collagen intensity, and maturation. Evidently, the polyphenols of

MFCE had contributed to the anti-inflammatory effect. The use of *Eleutherine indica* in the treated wound accelerates wound healing through the effect of the stimulation of released TGF-β which is bound to the fibroblast receptors and initiates collagen production (Upadhyay et al., 2013). Similar results were produced with *Polygonum cuspidatum* in the treated wound. The immunohistochemistry results showed that TGF-ß1 increased significantly in the group treated with plant extract on day 1, 3 and 7 after wounding (Wu et al., 2012). These results clearly indicate that the effect of MFCE in accelerating wound healing may be due to the effect of the rapid movement to the proliferative phase and the shortening of the inflammatory phase in order to enhance wound contraction.

It is well established that collagen formation is very important for tissue repair and remodeling, as it is a principle component of connective tissue and provides a structural skeleton for the regeneration of tissue enhanced tensile strength of the healing wound (Ponrasu, et al., 2014). Using the Masson's trichrome staining method, it was shown in this study that at day 15, the granulation tissue in the treated skin showed comparatively more collagen deposition and fibroblasts, and higher angiogenesis than in the untreated control group as reported in previous studies that used different compounds and/or plants such as 3-(2-chlorophenyl)-1-phenyl-propenomein, ethanol extract of *Bacopa monniera* (Abu-Al-Basal, 2010; Udegbunam et al., 2012; Murthy et al., 2013). Antioxidant, anti-inflammatory, and antimicrobial effects of polyphenols are responsible for the acceleration of wound healing that is facilitated by rapid wound closure and increased rate of epidermal regeneration (Xin et al., 2008; Lai et al., 2011). The MFCE is thus shown to stimulate matrix deposition and the resolution of tissue at the wound site, these effects are attributed to the high phenolic content of MFCE (Sakong et al., 2011). The underlying mechanisms of topical application of MFCE in the wound

area which are caused by the chemotactic influence of the plant extract may attract, increase cellular proliferation and contribute significantly to the healing process.

The process of wound healing is regulated and executed by a complex signaling network involving numerous growth factors. VEGF mediates angiogenesis and granulation tissue formation during wound healing and is expressed by many cells, including leukocytes and endothelial cells (Atiba et al., 2011). Increased VEGF expression has been reported to be associated with accelerated wound healing in model animals, while decreased expression occurred in poor wound healing (Manoj & Muragan., 2012; Pessoa et al., 2012). In this study, MFCE-HD increased the number of VEGF-positive cells in the wound tissue sections, which is suggested to be associated with increase in the growth factor expression. However, it is not certain how MFCE stimulates the expression of VEGF. It is possible that the furanocoumarins such as angelicin of MFCE is responsible for the effect, since it can regulate inflammation via inhibition of the MAPK/NF-κB pathways (Liu et al., 2013a). The finding also corroborates well with our earlier results of histopathology that revealed a complete re-generation of the epidermis, high collagen deposition, angiogenesis, and epithelialization in the groups treated with methanol fraction of *C. excavata* compared to the untreated control group. Therefore, findings from this investigation suggests that, topical application of methanol fraction of *C. excavata* could have the possibility to accelerate angiogenesis through angiogenic marker.

During the inflammation phase, various inflammatory cells like neutrophils, macrophages, fibroblasts, and endothelial cells produce a sudden burst of ROS in the site of the wound, which providing signaling and defense against invading microorganisms. Overproduction of ROS may also hinder the rate of wound healing by causing damage to surrounding cells. In fact, H_2O_2 reportedly causes fatal injury to

fibroblasts and blocks cell signaling by inhibiting epidermal growth factor (EGF) receptor internalization, in addition to the inhibiting keratinocyte migration (Phan et al., 2001). Thus, tissues must be protected from oxidative damage via intracellular and extracellular antioxidants, which enhance the healing process by reducing the damage to tissue and cell structures. Plant extracts such as MFCE possessing antioxidant properties can prevent oxidative damage to cells induced by wound injury, thereby promote healing (Ilango & Chitra, 2010; Ram et al., 2014). In this study, increased SOD, GPx and CAT, and decreased LPO activities were observed from wound tissue homogenates of HD-MFCE-treated groups. The reduced SOD as well as GPx activity in the homogenate wound obtained from the vehicle control group has been attributed to enhanced manufacture of reactive oxygen which could decrease the action of antioxidant enzymes (El-Razek et al., 2012). Similar results were reported from previous studies using *Plagiochasma appendiculatum* Lehm, where enhanced wound healing rate was attributed to decreased activity of LPO and increased activities of SOD and CAT (Singh et al., 2006). The inhibition of ROS production and LPO promotes fibroblast proliferation, neovascularization, and the healing process of wounds (Murthy et al., 2013).

Inflammatory cells invade the wound tissue within a few hours after injury, and their expression is strongly up-regulated during the inflammatory phase of healing. These pro-inflammatory cytokines are potent inducers of matrix metalloproteinases (MMP) synthesis in fibroblasts and inflammatory cells, resulting in inhibits collagen formation and hydroxyproline production, which are essential for the final part of the proliferative phase, thus impaired in wound healing (Lobmann et al., 2002; Wojdasiewicz et al.,2014). It is likely that the balance between protease and inhibitor concentrations plays a crucial part in successful wound healing (McCarty & Percival, 2013). The high concentrations of pro-inflammatory cytokines and proteases act as a positive feedback

loop involving increase inflammatory cells and releasing cytokines, which stimulate wound cells to secrete proteases that destroy tissue and prevent the wound from closing (Guo & DiPietro, 2010). These cytokines particularly stimulate of keratinocyte, granulation tissue formation, fibroblast proliferation, synthesis of collagen, and breakdown of ECM. The expressions of pro-inflammatory cytokine IL-6 and TNF-α are strongly up-regulated during the inflammatory phase of healing. The IL-6 promotes inflammation through monocyte and neutrophil chemotaxis and activation. One of the means to facilitate a scar less wound repair is to subdue the inflammatory cytokine cascade in the wound (Liu et al., 2013b). IL-10 appears to influence the wound-healing environment by decreasing the expression of pro inflammatory/profibrotic mediators, resulting in decreased recruitment of inflammatory cells to the wound by inhibiting the expression of specific chemokines, including macrophage inflammatory protein-1α (MIP-1α) and monocyte chemoattractant protein-1 (MCP-1) (Peranteau et al., 2008). In addition IL-10 has been shown that is responsible for inhibiting the production of MMPs family of metalloproteinase (Wojdasiewicz et al., 2014). In the present study, MFCE treatment in fact revealed down regulation of pro-inflammatory cytokines (IL-6 and TNF-α) while simultaneously increased the anti-inflammatory cytokines IL-10 concentration present at the wound site. These findings suggested that *C. excavata* regulates anti-inflammatory and proinflammatory cytokines and ultimately the systemic immune pathways associated with inflammation, allowing for faster tissue repair. The significant changes in cytokines levels are in accordance with other studies that linked decrease in pro-inflammatory cytokines level during healing of chronic wounds and the reduction in inflammatory state of the wound (Kapoor et al., 2004; Rosique et al., 2015).

In addition during the process of wound healing, COX-2 that is reported to be predominantly expressed during the inflammation process, and found to reduce as the

healing process starts. COX-2 is an inducible isoform of COX-2 and it is found to be rapidly induced in macrophages and endothelial cells by proinflammatory cytokines, may be responsible for the edema and vasodilation associated with inflammation (Muthukumar et al., 2014). Following gene expression analysis, down regulation of COX- 2 was observed from the wounds of rats treated with MFCE after 14 days, while there was upregulation in the wounds of untreated control. These findings were similar to the previous reports (Muthukumar et al., 2014; Yip et al., 2015), and it is believed that the reduction in the epidermal COX-2 plays an important physiological role in the wound healing.

Incidentally, *C. excavata* has a wide variety of secondary metabolites, such as coumarins, quercetin glycoside kaempferol and myricitin glucoside. All these metabolites displayed antioxidant, anti-inflammatory and antimicrobial effects (Calderón-Montaño et al., 2011; Krishnappa et al., 2014). Thus, the presence of these antioxidant, anti-inflammatory properties of MFCE, which in turn result from high phenolic content are proposed to be the prime mechanism in the facilitation of wound healing.

5.3.1. Analysis of BAX and BCL2 gene expression in healed wounds in rats

Apoptosis is the active process of eliminating cells, which plays a vital role in reducing inflammation by removing inflammatory cells and granulation tissue (Mutsaers et al., 1997). Neutrophils are the first cells that arrive at the wound site to eliminate microorganisms and initiate the inflammatory process. Neutrophils and other recruited inflammatory cells could generate ROS in the wound area. Overproduction of ROS at the wound site results in the induction of apoptosis of surrounding cells, including keratinocytes, through activation of apoptosis-related proteins such as Bax, Bad, and Bid were known to be pro apoptotic, whereas others, such as Bcl-2 and Bcl-xl

were shown to inhibit the apoptotic process (Cepero et al., 2001; Rouhollahi et al., 2015). Abnormal control of apoptosis in wound healing might be a crucial reason for delayed wound healing. BAX and BCL2 proteins play an important role in the apoptosis pathway in regulating wound healing. The expression of BAX protein increased similarly with increasing apoptosis and revealed a good compatible relationship with the wound healing process, whereas the expression of BCL2 protein declined apoptosis (Cui et al., 2003).

In this study, the Fold change ($2^{-\Delta\Delta CT}$) of the mRNA expression level of the BAX and BCL2 on the 14th day after wound, showed down-regulation in the expression of the BAX gene in the *C. excavata* treated group with fold change (3.25) and slight down-regulation in the reference drug group with fold change (1.65) when compared to the calibrator group. On the other hand significant up-regulation occurred in the BCL2 genes in the *C. excavata* treated groups with fold change (2.75891) and slight up-regulation in the reference drug group with fold change (1.33) when compared to the calibrator group. As the group with topical treatment of *C. excavata* at a dose of 200 mg/ml showed down regulation of the BAX gene expression, this may indicate that the wound-healing process was at a later stage; remodeling phase, while in the intrasite gel group it may be an indication that slight down apoptosis signals the end of the inflammatory phase of healing. This data also indicates that the apoptosis of granulation tissue begins at wound closure and decreases when the wound is healed or closed at about 95%. These findings confirm the results of a previous study by Rouhollahi et al., (2015) which suggested that a topical application of *Curcuma purpurascens* induced down regulation of BAX. Another study also showed that apoptosis and protein expression increased in both the inflammatory and proliferation stages of wound healing, but decreased during the remodeling stage. In addition, apoptosis plays a vital role to epidermal regeneration; decrease apoptosis-regulates the proliferation process or

it is associated with accelerated terminal differentiation of keratinocytes in the regenerated epidermis (Nagata et al., 1999). Another study by Kane & Greenhalgh, (2000) which suggested that an inverse relationship existed between BCL2 and p53. The expression of BCL2 was increased to allow cellular proliferation to occur at the beginning of the injury, whereas p53 expression decreased. Another study reported that increased apoptosis in diabetic wound may be due to persistent hyperglycemia due to oxidative stress (Bhan et al., 2013).

5.4. Evaluation of gastric ulcer activity

Gastric ulcers are caused by imbalance between the intrinsic protective mechanism (growth factors, mucous and bicarbonates) and offensive endogenous (increased acid and pepsin secretions) and exogenous (stress, use of cigarettes and alcohol, *Helicobacter pylori* and non-steroidal anti-inflammatory drugs) factors in the mucosa (Yu et al., 2014).

Today, different approaches for treating peptic ulcer are used to reducing the gastric acid secretion or re-enforcing the mucosal defense mechanism by increasing the mucus production protecting the surface epithelial cells or interfering with the prostaglandin E2 synthesis (Sumbul et al., 2011). A more recent approach to this includes proton pump inhibitors, histamine H2 receptor antagonists, drugs affecting the mucosal barrier and prostaglandin analog (Al Asmari et al., 2016). The drugs currently available in the market show limited efficacy against gastric ulcer diseases and their long-term use is associated with disturbing side effects such as arrhythmias, impotence, gynecomastia, osteoporosis, constipation and hematopoietic changes (Awaad et al., 2014). Thus, there is an urgent need for new antiulcer drugs, which includes herbal drugs. The advantage of the natural products is that they have fewer side effects, more effective and relatively less expensive than the commercial drugs.

Ligation of the pylorus is one of the most common models to study the anti-secretory efficacy of drugs on gastric juice secretions and mucus secretion due to excess production and accumulation of gastric acid (Gyawali et al., 2013; Zakaria et al., 2015). The ligation of the pyloric end of the stomach, which triggers the activity of the partial cells H+,K+ -ATPase by stimulation of pressure receptors in the antral gastric mucosa, causes an increase in acid-pepsin accumulation in the stomach, which are responsible for producing ulcers through auto-digestion of gastric mucosa and breakdown of the gastric mucosal barrier (Schubert, 2011; Zakaria et al., 2015). Moreover, pyloric ligation disturbs cytoprotective factors such as prostaglandin, cytokines, membranes lipid peroxidation, and endogenous glutathione, resulting in inducing tissue injury (muthuraman & Sood, 2010). Thus, inhibition of gastric acid over secretion is one approach to prevent gastric injuries. To investigate the anti-secretory effect of MFCE the pyloric ligation used in the rat model. It was shown that MFCE reduced the volume and total acidity of the gastric juice. Thus, we considered that the antiulcer effect of MFCE may partially be through its anti-secretory effect of gastric juices. Similarly, the observed reduction in gastric acidity due to the reported suppression of the activity of hydrogen/potassium adenosine triphosphates (ATPase) in the gastric parietal cells, or the blocking of the action of histamine on these cells (Mitra & Kesisoglou, 2013).

Gastric mucus is the first line of defense secreted by the mucus neck cells. It comprises of mucin-type glycoproteins that serve as a barrier to back diffusion of hydrogen ions and reducing stomach wall friction during peristalsis and can be detected by amounts of Alcian Blue binding, (Zakaria et al., 2011; Zakaria et al., 2014). In this study, the ability of *C.excavata* to enhance the gastric mucus wall secretion proves that it could be one of the potential mechanisms of the gastro protective effect exerted by MFCE. Similar results were observed in previous studies using *Melastoma*

malabathricum leaves, which showed gastro protective ability by significantly increasing mucus production (Zakaria et al., 2015).

Polyphenols, like coumarins and flavonoids inhibit H+/K+ - ATPase, decrease histamine and stimulate prostaglandin biosynthesis, it was postulated that these mechanisms of action may be responsible for the antiulcer activity of flavonoids (Bighetti et al., 2005; Sakat et al., 2012; Sharath et al., 2014).

Consumption of excessive alcohol frequently produces gastric mucosal injuries, which may progress to gastric cancer. Thus, the administration of absolute ethanol by gavage has been used as reproducible method to induce gastric damage in rats and to investigate the gastro protective effects of medicinal plants (Adinortey et al., 2013; Antonisamy et al., 2014). According to Shanmukha et al. (2013), necrotizing agents-induced gastric ulcers and the lesions were characterized by multiple haemorrhage red bands of different sizes along the longitudinal axis of the glandular stomach. This model is extensively used to screen drugs for cytoprotection. Ethanol rapidly penetrates into the gastric mucosa causes development of necrotic lesions in the gastric mucosa, damaged areas in the deep layers of the mucosa with necrosis by decreasing mucus and bicarbonate when it reaches the mucosa and results in the induce disturb the wall of blood vessels, inflammatory cell penetration, hypoxia and therefore overproductions of ROS. High concentrations of ROS cause reduction in the levels of major antioxidants, lipid peroxidation, and loss of epithelial cells in the stomach and cellular apoptosis that are classic features of alcohol damage (Vendramini-Costa et al., 2014; Li et al., 2013; Luo et al., 2013). As a model, ethanol-induced ulcer may not be appropriate or useful for the assessment of the usefulness of anti-secretory drugs or testing materials due to the absence of gastric acid secretion where acid secretion underlies the development of the ulcer.

Many studies have been done to investigate the gastro protection of different plants from ethanol induced gastric injuries, the protection being through the involvement of a different mechanism (Zheng et al., 2014; Miranda et al., 2015). However, in natural plant products there are several agents purported to be also effective in the treatment of gastric ulcers; among which are products from the *C. excavata* plant. Using the ethanol-induced rat gastric ulcer model to investigate MFCE ability in gastric protection and its possible mechanisms of actions, the pretreated groups with different doses of MFCE showed significant decrease in ulcer area and increased ulcer inhibition percentage, and this effect is attributable to the free radical scavenging properties and anti-inflammatory activity of the extract (Ishida et al., 2010). This in turn reduces neutrophil infiltration and strengthens the protection of gastric mucosa, resulting in an enhanced ulcer healing process. The data show that the MFCE did not present a dose-dependent response. Da Silva et al., (2013) stated that plant extracts can act as antioxidants or pro-oxidants, depending on the structure and composition of the different classes of polyphenols present in the extract.

Among the eminent gastric protection mechanisms is the gastric mucosa lining, which is known to compose of a jelly mixture of water and glycogen serves as the first line of defense to gastric lumen from the aggressive effects of necrotizing agents (Sidahmed et al., 2015). The efficiency of the mucosa membrane to protect the gastric walls highly depends on the equilibrium balance between the aggressive and the protective factors (Salga al., 2012). In our study, MFCE was shown to increase production of gastric mucus from mucous cells to form the first line of defense to protect the mucosal epithelial layer from damage by the ulcerogenic action of ethanol. In fact, treatment with the extract did not only increase mucus production by the gastric mucosal but also increased the pH of gastric juice that serve to curb development of gastric ulcers. Similar results were observed in previous studies (Halabi et al., 2014).

The contraction caused by ethanol in the circular muscles of the stomach can lead to mucosal compression at the site of the greatest mechanical stress, at the crests of mucosal folds leading to necrosis and ulceration (Mahmood et al., 2010). The enhanced flattening of the mucosa folds surface as a result of MFCE treatment could be attributed to circular muscle relaxations that resulted in more mucosal area exposure to the action of the MFCE at the same time reduce the volume of the gastric irritants in the rugal crest (Bardi et al., 2011). Thus, the mechanism of mucosa protection may be the anti-secretory activity of *C. excavata* leaves and its effect on enhancing mucin production.

The observed gastro-protective effect of these MFCE could be due to the restoration of the gastric mucosa by improving the continuous generation of COX-1, which catalyzes the rate-limiting step in the conversion of arachidonic acid to prostaglandins such as PGE2 (da Silva et al., 2013). Prostaglandins control gastric acid secretion, enhances gastric mucus and bicarbonate productions, promotes angiogenesis, inhibiting leukocyte recruitment, and therefore inhibit the free radical production and the inflammatory mediator release, thus play significant protective roles by conferring the gastric mucosal the facility to resist injury caused by noxious compounds (Da Silva et al., 2013; Balogun et al., 2014). The gastric mucosa with ulcers showed higher expression of PGE2 after MFCE treatment than those not treated. The result suggests that enhancement of PGE2 activity is another mechanism by which MFCE reduces ulceration in the gastric mucosa. Thus, it can be concluded that the gastroprotective effect is partly due to the coumarin and flavonoid in extract that is a stimulator of gastric prostaglandin and mucus production (Goel et al., 1997; Bighetti et al., 2005; Kumar et al., 2011; Sakat et al., 2012; Seigler et al., 2012). The findings of this study are supported by the results obtained through investigating the effect of *Decalepis hamiltonii* root polysaccharide as an antiulcer agent against ethanol induced gastric ulcer (Srikanta et al., 2010).

The histopathological evaluation of the gastric mucosa by H & E showed that MFCE inhibits leucocyte infiltration, hemorrhage, oedema and epithelial cell loss in rat gastric tissues with ethanol-induced gastric ulcer. Additionally, myriads of research have associated infiltration of neutrophils with the release of pro-inflammatory mediators (Olatunji et al., 2015). On the other hand, the released inflammatory mediators stimulate the production of potent ROS, which interfere with the healing process of gastric ulcers (Al-Rashdi et al., 2012). Therefore, the MFCE could probably play a role in inhibiting the infiltration of neutrophils in the gastric ulcer tissues, thus enhancing the healing process. In fact, the healing and lesion-inhibition effects of MFCE were suggested to be through its antioxidant and anti-inflammatory activities (Manosroi et al., 2004; Sakong et al., 2011). A similar notion of antiulcer drug activity by inhibiting neutrophil infiltration was reported (Salga et al., 2012; Wong et al., 2013).

The mucus layer lining of the gastric mucosa, acting as physical barrier against the aggressive effect of the gastric juice as well as reduces the damaging effect of oxidative stress. Periodic Acid–Schiff (PAS) staining is widely used in staining carbohydrates having high molecular weights, such as mucins, glycolipids, and glycoproteins (Kilcoyne et al., 2011). Thus, through using PAS the integrity of the mucus lining can easily be ascertained. Observation of PAS stained tissue sections showed a gradual increases mucus production in MFCE treated groups. Similar intense coloration was observed in the glandular epithelium of experimental ulcer tissues treated with different compounds (Devaraj et al., 2011; Salga et al., 2012). Therefore, the gastro protective effect of this plant might attribute to their activity in maintaining gastric mucus integrity.

The regulation of apoptosis in the tissues is complex, involving the activation of several apoptosis related proteins such as the Bcl-2 family, p53, and Fas and its ligand (FasL). Some members of the Bcl-2 family, such as Bax, Bad or Bid, are known to be proapoptosis, whereas the other members, such as Bcl-2 and Bcl-xl are antiapoptosis (Cepero et al., 2001). The susceptibility of cells to apoptosis depends on the balance between apoptosis-promoting and apoptosis-suppressing (Goel et al., 1997). It was found that apoptosis is regulated by ROS production. PGE2 was able to inhibit apoptosis and stimulate the expression of Bcl-2 (Alibek et al., 2014). One of the mechanisms in gastric ulceration is induction of apoptosis of the gastric mucosal cells (Rudin et al., 1997; Fornai et al., 2011). Thus, another means of inhibition of gastric ulceration is by the prevention of premature mucosal cell apoptosis. It was reported earlier that omperazol protect mucosa from imjuries by acting as potent antioxidant and antiapoptotic agent. Our results showed that MFCE caused down-regulation of BAX, a pro-apoptotic protein expression in the rat gastric tissues with ulcer. In untreated rats, the BAX protein is up-regulated. This suggests that MFCE retards premature cell death in the gastric mucosa, an effect that is strongly associated with the gastro protective property of the extract through anti-apoptotic process.

HSP-70 protein from the heat shock protein family is ubiquitously present in mammalian cells and this protein serves to defend cells from oxidative stress damage. In the cell, the HSP-70 preserves the functional structure of normal proteins while repairing or removing damaged proteins. In the gastric mucosa, ROS generation by ethanol impedes expression of HSP70 making the mucosal layer susceptible to ulcerative damage. HSP70 inductions protected rats against ethanol induced gastric mucosal damages, Thus HSP-70 may contribute in gastroprotective (Emily et al., 2012; Al Rashdi et al., 2012). However, treatment with MFCE increased expression of HSP70 in the gastric mucosa with ethanol-induced ulcer, which suggests that this effect is one

of the many facets of the gastro protective properties of MFCE. Similar findings have been reported by other authors using different compounds and plant extracts (Shichijo et al., 2003; Chen et al., 2005; Al Batran et al., 2013a; Al Batran et al., 2013b).

TGF-β is one of the most important growth factors that have a healing effect on gastric ulcer through induction of cell migration, cell growth and differentiation, and increased epithelial cells proliferation. In fact, TGF-β exists in three isoforms (1, 2, and 3), and is said to play a crucial role in complex growth regulatory activities, inflammation, and host immunity on virtually all types of cells (Chin et al., 2004; Clark & Coker, 1998; Wahl, 2002). As expected, in the ulcer group, a low expression of TGF-β was observed due to poor cellular proliferation. Conversely, there was an increased expression of TGF-β in the omeprazole treated and MFCE treated groups. These findings were similar to those reported by (Dhiyaaldeen et al., 2014) following administration of HPTP chalcone in rats having indomethacin induced ulcer, the immunoreactive effect of TGF-β enhances proliferation of epithelial cells in the gastric glands (Tanigawa et al., 2005; Walsh et al., 2008).

To further ascertain the gastroprotective property of MFCE, its effect on antioxidant enzymes and scavenging of free radicals from the oxidative stress induced by ethanol was determined. Reactive Oxygen Species (ROS) such as hydroxyl radical and superoxide radical anion are generated at a low rate in normal cellular metabolism and these oxidative radicals are scavenged by the endogenous antioxidant defense system of the body (Hiruma-Lima et al., 2006), namely the SOD, GPx, and CAT. Superoxide dismutase-mediated catalyse the superoxide radical anion (O2•−) into less noxious hydrogen peroxide (H_2O_2). This represents the first line of antioxidant defense by catalase through the transformation of H_2O_2 into water (La Casa et al., 2000; Tripathi & Chandra, 2009). Since the accumulation of ROS increases lipid peroxidation (LPO), the

LPO content in the gastric mucosa can be used as a biomarker for development of ROS-mediated gastric mucosal lesions which has a critical role in the pathogenesis of gastric ulceration (Agarwal et al., 2012). ROS are reportedly capable of oxidizing cellular lipids that contain unsaturated fatty acids with more than one double bond are said to be more susceptible to the oxidizing action of free radicals (Alzoghaibi, 2013), resulting in the generation of MDA as one of the major secondary oxidation products. The generated MDA is shown to integrate to the cellular DNA, causing cytotoxic and mutagenic effects (Ayala et al., 2014). Thus, lipid peroxidation assay evaluates the oxidative degradation of lipids, which caused the disruption of cellular membranes. Thus, in pathological conditions like gastric ulceration, ROS may be produced in excess and disrupt the delicate balance between ROS and endogenous antioxidant enzymes (Agarwal et al., 2012). In this study, MFCE increased the activity of ROS scavenging enzymes SOD, GPx and CAT and decreased LPO contents in the gastric mucosa, further confirming that the methanolic fraction is gastro protective to the development of ethanol-induced ulcers by scavenge the free radicals resulting from the oxidative stress induced by ethanol through antioxidant mechanism. This scavenging ability of *C. excavata* comes from their high phenolic and flavonoid contents. In similar research, numerous medicinal plants have been employed for their antioxidant potential and investigated for their gastro protective abilities (Khan& Khan, 2013; Hussein et al., 2014).

One of the important findings in this study was that the level of pro-inflammatory factor TNF-α and IL-6 significantly decreased in groups pretreated with MFCE, which increased the level of anti-inflammatory cytokine IL-10 and TGF-β1. These results indicate that the immunomodulatory effect of *C. excavata* might be affected through the inflammatory mediator. TNF-α being a major pro-inflammatory cytokine produced by macrophages attract neutrophils to the site of gastric mucosal injury as well as stimulate

inducible nitric oxide expression (Wei et al., 2003; Martin & Wallace, 2006). IL-6 is shown to mediate immune response and acute inflammation. IL-6 activates granulocytes, which in turn trigger a stress response in injured tissue (Kishimoto, 2005; Mei et al., 2012). The decreasing levels of TNF-α and IL-6 in pretreated group are a result of the immune suppressor effect on elevated TGF-β1 and IL-10 (Taylor et al., 2006; Kamarazaman et al., 2012). Sabat et al. (2010) suggested that IL-10 has the ability to suppress the inflammatory response and inhibit TNF-α production. The results of our study showed that MFCE pre-treatment inhibited depletion of IL-10 and elevation of TNF- α and IL-6 level, which showed its anti-inflammatory effect. These activities are key in the prevention of gastric ulcer as reported earlier (Swarnakar et al., 2005; Zhou et al., 2014).

CHAPTER 6: CONCLUSION AND FURTHER WORK

The current study revealed the *in vitro* and *in vivo* bioactivity of *C. excavata* which includes: radical scavenging power, enhancement of cellular proliferation and migration of cells, toxicological implications, and gastro protective wound-healing potentials. The MFCE leaves contain pharmacologically active compounds which include: flavonoids and phenolic compounds, which are good antioxidants. In addition, remarkable antioxidant activities were demonstrated by the bioactive methanol fraction through radical scavenging and ferric reducing abilities as well the total phenolic and flavonoid contents. The MFCE leaves did not exhibit obvious toxicity at concentrations as high as 5000mg/kg body weight. Thus the *C. excavata* leaves have potent antioxidant activity and is presumed safe to be used orally as health-promoting product at low to moderate doses.

The methanolic fraction was effective in enhancing wound healing *in vitro* through enhanced proliferation and migration of fibroblast cells. Furthermore, topical applications of methanolic fraction for 14 days enhanced wound healing through overall decrease in wound size, improved wound contraction, reduced inflammation, angiogenesis, increased collagen synthesis and fibroblast synthesis, decreased activities of lipid peroxidase and increased SOD, CAT, and GPx activities, as well as modulation of inflammatory cytokines and inflammatory mediators such as TNF-α, IL-6, COX-2 and IL10. VEGF and TGF-β1 expression in the MFCE-treated group were dramatically increased and higher than in the control.

In addition, this study also showed that the gastro-protective properties of MFCE are elicited through a number of mechanisms including, inhibition of gastric juice and enhancement of gastric mucus secretions, elevation of gastric juice pH, stimulation of antioxidant enzyme activity, PGE2, and up-regulation of HSP-70 and TGF-β protein

expressions in the gastric mucosa as well as increased secretion of interleukin IL-10 and decreased secretion of interleukin IL-6, and TNF-α. Based on the findings of this work, methanolic fraction has a potential to be developed into a therapeutic compound for the treatment of gastric ulcers. In either ulcer or wound healing, methanolic administration was showed downregulation of BCL2 associated X protein (BAX); suggest that MFCE provoked apoptosis that is responsible for protecting gastric walls and skin from damage. Our results indicate that *C. excavata* possess anti-inflammatory potential, wound healing activity and gastro protective effect.

The results obtained from this study suggest that *C. excavata* can be used as source of antioxidants and supplement in nutritional and pharmaceutical industry, especially the methanol fraction can serve as a potential source of drug discovery for treatment of wounds and gastritis conditions. Further studies are needed to confirm the potential properties for the active constituents identified in this study such as bioavailability, pharmacokinetics and other pharmacological evaluations.

Recommendations for future research

- Similar study should be conducted using other wound models such as incision; dead space wound model to determine the effectiveness of this fraction in other models of wound healing in diabetic condition.
- There is need to further determine the major active compounds in MFCE so the full potential of the extract as a therapeutic compound can be realized.
- Further studies are needed to understand and validate on how this plant can contribute to maintenance of human physiological health, particularly its stability and bioavailability in human

- Additional studies are essential to confirm the potential properties for active constituents identified in this study such as bioavailability, pharmacokinetics and other pharmacological evaluations.

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APPENDIX A: PREPARATION OF REAGENTS AND EXPERIMENTAL METHODS

1. Ferric reducing power (FRAP) Reagents: Iron (II) sulfate hepatohydrate ($FeSO_4 \cdot 7H_2O$), Sodium acetate trihydrate buffer ($C_2H_3O_2N_9 \cdot 3H_2O$), 2, 4, 6-tris (2-pyridyl)-s-triazine (TPTZ) and 98% $FeCl_3 \cdot 6H_2O$

Preparation of reagents 0.0278 g of $FeSO_4 \cdot 7H_2O$ was dissolved in 100 ml dH_2O and 0.0775 g of acetate buffer was dissolved in 25 ml dH_2O mixed previously with 0.4 ml glacial acetic acid. TPTZ (0.00781 g) was dissolved in 2.5 ml dH_2O mixed previously with 0.1 ml (1M HCL). Finally, 0.0135g $FeCl_3 \cdot 6H_2O$ was dissolved in 2.5 ml dH_2O. The freshly prepared acetate buffer, TPTZ and $FeCl_3 \cdot 6H_2O$ solutions were mixed and vortexed to obtain the ready to use FRAP reagent.

2. DPPH free radical scavenging assay Reagents: 2, 2-diphenyl-1-picrylehydrazyl ($C_{18}H_{12}N_5O_0$) solution.

Preparation of reagent: DPPH solution was prepared by dissolving 0.001972 g of DPPH in 50 ml of absolute ethanol then stirred until the DPPH was completely dissolved.

3. PREPARATION OF 2% AGAROSE GEL ELECTROPHORESIS

Dissolve (1gm) of agarose in 100ml of 1x Tris-acetate EDTA buffer, then add 1µl of ethidium bromide to the mixture and pour on the gel boat to the electrophoresis system. After gel is solidified, load RNA and run the electrophoresis at 80 volts to migrate the RNA bands 28S and 18S.

4. TISSUE PROCESSING AND STAINING

a. PREPARATION OF TISSUE FOR PARAFFIN FIXATION

For paraffin, processed tissue the most usual fixative is formalin. Use 10% formalin for fixation to prepare 10% formalin; mix 100ml of Formaldehyde 40%, 900ml Distilled water and adjusted the PH at (7 - 7.4) by adding the tablet of PBS and mix well.

b. AUTOMATED TISSUE PROCESSING (LEICA TP1020):

FIXATION: (10% buffered formalin I for 1 hour and 10% buffered formalin II for 1 hour)

DEHYDRATION: The traditional method of dehydration –removing the water from the tissue - is the use of ascending concentrations of alcohols. Dehydration usually commences using 70% ethanol, progressing to absolute alcohol.

- 70% ethanol for 1 hour.
- 95% ethanol for 1 hour.
- 95% ethanol for 1 hour
- 95% ethanol for 1 hour
- First absolute ethanol for 1 hour.
- Second absolute ethanol 1½ hours.

CLEANING: The next step is called "clearing" and consists of removal of dehydrating fluids (alcohols) and paraffin wax with a substance that will be miscible with the embedding medi-um (paraffin). The commonest clearing agent is xylene.

- Cleaning Alcohol : Xylene (1:1) for 1 hour
- Xylene I for 1½ hours
- Xylene II for 1½ hours

INFILTRATION

- Infiltration Paraffin wax I for 1½ hours
- Paraffin wax II for 1½ hours

c. HEMATOXYLIN-EOSIN (H&E) STAINING PROTOCOL

MAIN STEPS OF STAINING

- De-waxing with xylene
- Re-hydration
- Staining
- Dehydration with xylene
- Mounting

- **STAINING WITH HAEMATOXYLIN & EOSIN:**

STEP	SOLUTIN	TIME(MINUTE)
De-waxing	Xylene I	3 min
	Xylene II	3 min
Re-hydration	Absolute alcohol	2 min
	95% alcohol I	2 min
		2 min
	95% alcohol II	2 min
	70% alcohol	
Bring section to water	Running tap water	3 min
Staining	Hematoxylin stain	10 min
	Running tap water	until excess color wash off
Differentiation	0.5% acid alcohol	2-3 sec
	Running tap water	2-3 min
		2 sec
	2% sodium acetate	2-3 min
		2-3 sec
	Running tap water	
	Rinse in 80%alcohol	
Staining	Eosin stain	5 min
Dehydration	95% alcohol I	5 sec
	95% alcohol II	2 min
		2 min
	Absolute ethanol I	2 min
	Absolute ethanol II	
Cleaning	Xylene I	2 min
	Xylene II	2 min
		1 min
	Xylene III	

Mounting with DPX (DPX mounting media and cover the section with cover slip.

STAINING WITH PERIODIC ACID SCHIFF BASE (PAS):

STEP	SOLUTIN	TIME(MINUTE)
De-waxing	Xylene I	3 min
	Xylene II	3 min
Re-hydration	Absolute alcohol	2 min
	95% alcohol I	2 min
		2 min
	95% alcohol II	2 min
	70% alcohol	
Bring section to water	Running tap water	3 min
Staining	Periodic acid	3 min
	Running tap water	until excess color wash off
	Schiff reagent	15 min
	Running tap water	2-3 min
Staining	Hematoxylin stain	1-2 min
Dehydration	95% alcohol I	5 sec
	95% alcohol II	2 min

	Absolute ethanol I	2 min
		2 min
	Absolute ethanol II	
Cleaning	Xylene I	2 min
	Xylene II	2 min
		1 min
	Xylene III	

Mounting with DPX (DPX mounting media and cover the section with cover slip.

STAINING WITH MASSON'S TRICHOM STAIN (MT):

STEP	**SOLUTIN**	**TIME(MINUTE)**
De-waxing	Xylene I	3 min
	Xylene II	3 min
Re-hydration	Absolute alcohol	2 min
	95% alcohol I	2 min
		2 min
	95% alcohol II	2 min
	70% alcohol	
Bring section to water	Running tap water	3 min
Staining	Bouins solution	1 hour
	Running tap water	until excess color wash off
	Weigers iron hematoxylin	10 min
	Rinse in distilled water	
	Biebrich scarlet-acid fuchsion	2 min
	Rinse in distilled water	
	Phosphomolybdic-phosphotungstic acid	15 min
	Light green	1 min
	Rinse in distilled water	
	Glacial acetic solution	3 min
Dehydration	95% alcohol I	5 sec
	95% alcohol II	2 min
		2 min
	Absolute ethanol I	2 min
	Absolute ethanol II	
Cleaning	Xylene I	2 min
	Xylene II	2 min
		1 min
	Xylene III	

Mounting with DPX (DPX mounting media and cover the section with cover slip.

APPENDIX B: MANUFACTURER INSTRUCTION OF KITS AND PROCEDURES

1. PGE2 (CAYMAN PGE2 ASSAY ELISA KIT; CAT# 400144)

5. Prepare SPE (C-18) columns by rinsing with 5 ml methanol followed by 5 ml deionized water. Do not allow the SPE Cartridge (C-18) to dry.
6. Apply the sample to the SPE Cartridge (C-18) and allow the sample to completely enter the packing material.
7. Wash the column with 5 ml deionized water. Discard the wash.
8. Elute the PGE_2 from the column with 5 ml ethyl acetate containing 1% methanol. Higher recovery and better reproducibility may be obtained if the sample is applied and eluted by gravity. The wash steps may be performed under vacuum or pressure.
9. Evaporate the ethyl acetate to dryness under a stream of nitrogen. It is very important that all of the organic solvent be removed as even small quantities will adversely affect the EIA.
10. To resuspend the sample, add 500 µl EIA Buffer. Vortex. It is common for insoluble precipitate to remain in the sample after addition of EIA Buffer; this will not affect the assay. This sample is now ready for use in the EIA.
11. Use 50 µl of the resuspended sample for scintillation counting.

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

PGE_2 Express EIA Standard

Reconstitute the contents of the PGE_2 Express EIA Standard (Item No. 400144) with 1.0 ml of EIA Buffer. The concentration of this solution (the bulk standard) will be 10 ng/ml. Stored at 4°C; this standard will be stable for up to four weeks.

NOTE: If assaying culture medium samples that have not been diluted with EIA Buffer, culture medium should be used in place of EIA Buffer for dilution of the standard curve.

To prepare the standard for use in EIA: Obtain eight clean test tubes and number them #1 through #8. Aliquot 800 µl EIA Buffer to tube #1 and 500 µl EIA Buffer to tubes #2-8. Transfer 200 µl of the bulk standard (10 ng/ml) to tube #1 and mix thoroughly. The concentration of this standard, the first point on the standard curve, will be 2 ng/ml (2,000 pg/ml). Serially dilute the standard by removing 500 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than 24 hours.

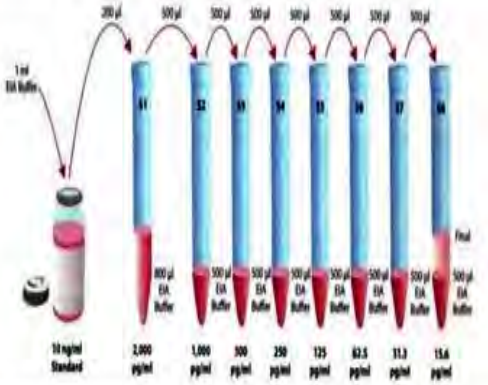

Figure 5. Preparation of the PGE_2 standards

Performing the Assay

Pipetting Hints

- Use different tips to pipette each reagent.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Addition of the Reagents

1. EIA Buffer

Add 100 µl EIA Buffer to Non-Specific Binding (NSB) wells. Add 50 µl EIA Buffer to Maximum Binding (B_0) wells. If culture medium was used to dilute the standard curve, substitute 50 µl of culture medium for EIA Buffer in the NSB and B_0 wells (*i.e.*, add 50 µl culture medium to NSB and B_0 wells and 50 µl EIA Buffer to NSB wells).

2. Prostaglandin E_2 Express EIA Standard

Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. Samples

Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. Prostaglandin E_2 Express AChE Tracer

Add 50 µl to each well *except* the Total Activity (TA) and the Blank (Blk) wells.

5. Prostaglandin E_2 Express Monoclonal Antibody

Add 50 µl to each well *except* the Total Activity (TA), the Non-Specific Binding (NSB), and the Blank (Blk) wells.

Well	EIA Buffer	Standard/ Sample	Tracer	Antibody
Blk	-	-	-	-
TA	-	-	5 µl (at devl. step)	-
NSB	100 µl	-	50 µl	-
B_0	50 µl	-	50 µl	50 µl
Std/Sample	-	50 µl	50 µl	50 µl

Table 1. Pipetting summary

Incubation of the Plate

Cover each plate with plastic film (Item No. 400012) and incubate 60 minutes at room temperature on an orbital shaker.

Development of the Plate

1. Reconstitute Ellman's Reagent immediately before use (20 ml of reagent is sufficient to develop 100 wells):

   100 dtn vial Ellman's Reagent (96-well kit; Item No. 400050): Reconstitute with 20 ml of UltraPure water.

   OR

   250 dtn vial Ellman's Reagent (480-well kit; Item No. 400050): Reconstitute with 50 ml of UltraPure water.

NOTE: Reconstituted Ellman's Reagent is unstable and should be used the same day it is prepared; protect the Ellman's Reagent from light when not in use. Extra vials of the reagent have been provided should a plate need to be re-developed or multiple assays run on different days.

2. Empty the wells and rinse five times with Wash Buffer.
3. Add 200 µl of Ellman's Reagent to each well.
4. Add 5 µl of tracer to the Total Activity wells.
5. Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark. This assay typically develops (*i.e.*, B_0 wells ≥0.3 A.U. (blank subtracted)) in 60-90 minutes.

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Remove the plate cover being careful to keep Ellman's Reagent from splashing on the cover. *NOTE: Any loss of Ellman's Reagent will affect the absorbance readings. If Ellman's Reagent is present on the cover, use a pipette to transfer the Ellman's Reagent into the well. If too much Ellman's Reagent has splashed on the cover to easily redistribute back into the wells, wash the plate three times with wash buffer and repeat the development with fresh Ellman's Reagent.*
3. Read the plate at a wavelength between 405 and 420 nm. The absorbance may be checked periodically until the B_0 wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the B_0 wells are in the range of 0.3-1.0 A.U. (blank subtracted). If the absorbance of the wells exceeds 1.5, wash the plate, add fresh Ellman's Reagent and let it develop again.

ANALYSIS

Many plate readers come with data reduction software that plot data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as either $\%B/B_0$ *versus* log concentration using a four-parameter logistic fit or as logit B/B_0 *versus* log concentration using a linear fit. *NOTE: Cayman has a computer spreadsheet available for data anaylsis. Please contact Technical Service or visit our website (www.caymanchem.com/analysis/eia) to obtain a free copy of this convenient data analysis tool.*

Calculations

Preparation of the Data

The following procedure is recommended for preparation of the data prior to graphical analysis.

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B_0 wells.
3. Subtract the NSB average from the B_0 average. This is the corrected B_0 or corrected maximum binding.
4. Calculate the B/B_0 (Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B_0 (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain $\%B/B_0$ for a logistic four-parameter fit, multiply these values by 100.)

NOTE: The total activity (TA) values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool; the corrected B_0 divided by the actual TA (10X measured absorbance) will give the % Bound. This value should closely approximate the % Bound that can be calculated from the ***Sample Data*** *(see page 28). Erratic absorbance values and a low (or no) % Bound could indicate the presence of organic solvents in the buffer or other technical problems (see page 32 for* ***Troubleshooting****).*

2. **SOD (CAYMAN, CAT#. 706002)**

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of SOD standards and samples to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 19).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
B	B	B	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
C	C	C	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
D	D	D	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
E	E	E	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
F	F	F	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
G	G	G	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
H	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41

A-G = Standards
S1-S41 = Sample Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 230 µl in all the wells.
- It is not necessary to use all the wells on the plate at one time.
- The assay temperature is 25°C.
- All reagents except samples and Xanthine Oxidase must be equilibrated to room temperature before beginning the assay.
- It is recommended that the samples and SOD standards be assayed at least in duplicate.
- Monitor the absorbance at 440-460 nm using a plate reader.

Standard Preparation

Dilute 20 µl of the SOD Standard (Item No. 706005) with 1.98 ml of Sample Buffer (dilute) to obtain the SOD stock solution. Take seven clean glass test tubes and mark them A-G. Add the amount of SOD stock and Sample Buffer (dilute) to each tube as described in Table 1 on page 12.

Tube	SOD Stock (µl)	Sample Buffer (µl)	Final SOD Activity (U/ml)
A	0	1,000	0
B	20	980	0.025
C	40	960	0.05
D	80	920	0.1
E	120	880	0.15
F	160	840	0.2
G	200	800	0.25

Table 1. Superoxide Dismutase standards

Performing the Assay

1. **SOD Standard Wells** - add 200 µl of the diluted Radical Detector and 10 µl of Standard (tubes A-G) per well in the designated wells on the plate (see sample plate format, Figure 2, page 10).
2. **Sample Wells** - add 200 µl of the diluted Radical Detector and 10 µl of sample to the wells. *NOTE: If using an inhibitor, add 190 µl of the diluted Radical Detector, 10 µl of inhibitor, and 10 µl of sample to the wells. The amount of sample added to the well should always be 10 µl. Samples should be diluted with Sample Buffer (dilute) or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 10,000 to bring the enzymatic activity to fall within the standard curve range.*
3. Initiate the reactions by adding 20 µl of diluted Xanthine Oxidase to all the wells you are using. Make sure to note the precise time you started and add the Xanthine Oxidase as quickly as possible. *NOTE: If assaying sample backgrounds, add 20 µl of Sample Buffer instead of Xanthine Oxidase.*
4. Carefully shake the 96-well plate for a few seconds to mix. Cover with the plate cover.
5. Incubate the plate on a shaker for 20 minutes at room temperature. Read the absorbance at 440-460 nm using a plate reader.

ANALYSIS

Calculations

1. Calculate the average absorbance of each standard and sample. If assayed, subtract sample background absorbance from the sample.
2. Divide standard A's absorbance by itself and divide standard A's absorbance by all the other standards and samples absorbances to yield the linearized rate (LR) (*i.e.*, LR for Std A = Abs Std A/Abs Std A; LR for Std B = Abs Std A/Abs Std B).
3. Plot the linearized SOD standard rate (LR) (from step 2 above) as a function of final SOD Activity (U/ml) from Table 1. See Figure 3 (on page 14) for a typical standard curve.
4. Calculate the SOD activity of the samples using the equation obtained from the linear regression of the standard curve substituting the linearized rate (LR) for each sample. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. SOD activity is standardized using the cytochrome c and xanthine oxidase coupled assay

$$\text{SOD (U/ml)} = \left[\left(\frac{\text{sample LR - y-intercept}}{\text{slope}}\right) \times \frac{0.23\ \text{ml}}{0.01\ \text{ml}}\right] \times \text{sample dilution}$$

Performance Characteristics

Precision:

When a series of 60 SOD standard measurements were performed on the same day, the intra-assay coefficient of variation was 3.2%. When a series of 60 SOD standard measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 3.7%.

Assay Range:

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 0.025-0.25 units/ml SOD.

3. CAT (CAYMAN CAT#. 707002)

Standard Preparation

1. Preparation of the Formaldehyde Standards - Dilute 10 µl of Catalase Formaldehyde Standard (Item No. 707014) with 9.99 ml of diluted Sample Buffer to obtain a 4.25 mM formaldehyde stock solution. Take seven clean glass test tubes and mark them A-G. Add the amount of formaldehyde stock and diluted Sample Buffer to each tube as described in Table 1 (below).

Tube	Formaldehyde (µl)	Sample Buffer (µl)	Final Concentration (µM formaldehyde)*
A	0	1,000	0
B	10	990	5
C	30	970	15
D	60	940	30
E	90	910	45
F	120	880	60
G	150	850	75

Table 1

*Final formaldehyde concentration in the 170 µl reaction.

Performing the Assay

1. **Formaldehyde Standard Wells** - Add 100 µl of diluted Assay Buffer, 30 µl of methanol, and 20 µl of standard (tubes A-G) per well in the designated wells on the plate (see sample plate format, Figure 1, page 12).
2. **Positive Control Wells (bovine liver CAT)** - Add 100 µl of diluted Assay Buffer, 30 µl of methanol, and 20 µl of diluted Catalase (Control) to two wells.
3. **Sample Wells** - Add 100 µl of diluted Assay Buffer, 30 µl of methanol, and 20 µl of sample to two wells. To obtain reproducible results, the amount of CAT added to the well should result in an activity between 2-35 nmol/min/ml. When necessary, samples should be diluted with diluted Sample Buffer or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 100,000 to bring the enzymatic activity to this level.
4. Initiate the reactions by adding 20 µl of diluted Hydrogen Peroxide to all the wells being used. Make sure to note the precise time the reaction is initiated and add the diluted Hydrogen Peroxide as quickly as possible.
5. Cover the plate with the plate cover and incubate on a shaker for 20 minutes at room temperature.
6. Add 30 µl of diluted Potassium Hydroxide to each well to terminate the reaction and then add 30 µl of Catalase Purpald (Chromagen) (Item No. 707017) to each well.
7. Cover the plate with the plate cover and incubate for 10 minutes at room temperature on the shaker.
8. Add 10 µl of Catalase Potassium Periodate (Item No. 707018) to each well. Cover with plate cover and incubate five minutes at room temperature on a shaker.
9. Read the absorbance at 540 nm using a plate reader.

4. GPX (CAYMAN CAT#. 703102)

Performing the Assay

1. **Background or Non-enzymatic Wells** - add 120 µl of Assay Buffer and 50 µl of co-substrate mixture to three wells.
2. **Positive Control Wells (bovine erythrocyte GPx)** - add 100 µl of Assay Buffer, 50 µl of Co-Substrate Mixture, and 20 µl of diluted GPx (control) to three wells.
3. **Sample Wells** - add 100 µl of Assay Buffer, 50 µl of Co-Substrate Mixture, and 20 µl of sample to three wells. To obtain reproducible results, the amount of GPx added to the well should cause an absorbance decrease between 0.02 and 0.135/min. When necessary, samples should be diluted with Sample Buffer or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 10,000 to bring the enzymatic activity to this level. ***NOTE:*** *The amount of sample added to the well should always be 20 µl. To determine if an additional sample control should be performed see the* ***Interferences*** *section (page 14).*
4. Initiate the reactions by adding 20 µl of Cumene Hydroperoxide to all the wells being used. Make sure to note the precise time the reaction is initiated and add the Cumene Hydroperoxide as quickly as possible.
5. Carefully shake the plate for a few seconds to mix.
6. Read the absorbance once every minute at 340 nm using a plate reader to obtain at least 5 time points. ***NOTE:*** *The initial absorbance of the sample wells should not be above 1.2 or below 0.5.*

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5. LPO (CAYMAN CAT#. 705003)

Standard Preparation

Take 24 clean test tubes (glass or polypropylene) and mark them A-H in triplicate. Aliquot the Lipid Hydroperoxide Standard (HP) (Item No. 705014) and chloroform-methanol mixture to each tube as described in Table 1.

Tube	HP Standard (µl)	$CHCl_3$-CH_3OH (µl)	Final HP* (nmol)
A	0	950	0
B	10	940	0.5
C	20	930	1.0
D	30	920	1.5
E	40	910	2.0
F	60	890	3.0
G	80	870	4.0
H	100	850	5.0

Table 1.

*This is the final amount of hydroperoxide in the assay tube.

Performing the Assay

1. Add 500 µl† of the chloroform extract of each sample to appropriately labeled glass test tubes. *NOTE: Avoid any transfer of water from the extract.*
2. Aliquot 450 µl of chloroform-methanol solvent mixture to the sample test tubes.
3. Prepare the Chromogen by mixing equal volumes of FTS Reagent 1 and FTS Reagent 2 in a test tube and vortex. The volume of Chromogen to be prepared is dependent on the number of tubes assayed. Calculate 50 µl for each tube (*e.g.*, 24 x 50 µl for the tubes of the standard curve).
4. Add 50 µl of the freshly prepared Chromogen to each assay tube and mix well by vortexing. Close the test tubes tightly with polypropylene caps.
5. Keep the assay tubes at room temperature for five minutes.

NOTE: There are two methods of completing this assay, either using a spectrophotometer to test each sample separately (see step 6) or when using the glass 96-well plate, the values can be read with a plate reader (see steps 7-9).

6. Measure the absorbance of each tube at 500 nm using either glass or quartz 1 ml cuvettes. Use chloroform-methanol solvent mixture for blank if the spectrophotometer requires it.

OR

7. Transfer 300 µl from each tube into the 96-well plate following the configuration shown in Figure 1. (There is no specific pattern for using the wells on the plate. A typical layout of standards and samples to be measured in duplicate is given on page 10.) To avoid evaporation from the wells, you may cover the plate with aluminum foil as you aliquot the samples. *NOTE: Do <u>not</u> use plastic plate covers, the solvent will dissolve the cover. Do <u>not</u> shake the plate on the table top, it will scratch the bottom of the plate.*
8. Read the absorbance at 500 nm using a 96-well plate reader.
9. The 96-well plate can be reused, see **Pre-Assay Preparation** (see page 8) for instructions on how to clean the plate.

NOTE: The color is stable for two hours. If the test tubes are not closed tightly, evaporation of solvent will result in change of absorbance.

†Volume of the extract used for the assay can be changed depending on the concentration of hydroperoxide. However, adjust the volume of the chloroform-methanol solvent mixture accordingly to a final volume of 950 µl before the addition of the chromogen.

6. IL-6 ELISA KIT (CUSABIO, CAT#. ER3TNFA)

1. **Wash Buffer** If crystals have formed in the concentrate, warm up to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to prepare 500 ml of Wash Buffer.
2. **Standard** Reconstitute the **Standard** with 1.0 ml of **Sample Diluent**. This reconstitution produces a stock solution of 20 pg/ml. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making serial dilutions. The undiluted standard serves as the high standard (20 pg/ml). The **Sample Diluent** serves as the zero standard (0 pg/ml).
3. **Biotin-antibody** Dilute to the working concentration specified on the vial label using **Biotin-antibody Diluent**(1:100), respectively.
4. **HRP-avidin** Dilute to the working concentration specified on the vial label using **HRP-avidin Diluent**(1:100), respectively.

Precaution: The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.

SAMPLE COLLECTION AND STORAGE

- **Cell Culture Supernates** Remove particulates by centrifugation and assay immediately or aliquot and store samples at -20° C. Avoid repeated freeze-thaw cycles.
- **Serum** Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 g. Remove serum and assay immediately or aliquot and store samples at -20° C. Avoid repeated freeze-thaw cycles.
- **Plasma** Collect plasma using citrate, EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at -20° C. Avoid repeated freeze-thaw cycles.

Note: Grossly hemolyzed samples are not suitable for use in this assay.

ASSAY PROCEDURE

1. Add 100µl of Standard, Blank, or Sample per well. Cover with the adhesive strip. Incubate for 2 hours at 37° C.
2. Remove the liquid of each well, don't wash.
3. Add 100µl of **Biotin-antibody** working solution to each well. Incubate for 1 hour at 37°C. **Biotin-antibody** working solution may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.
4. Aspirate each well and wash, repeating the process three times for a total of three washes. Wash by filling each well with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Add 100µl of **HRP-avidin** working solution to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37° C.
6. Repeat the aspiration and wash three times as step 4.
7. Add 90µl of **TMB Substrate** to each well. Incubate for 30 minutes at 37°C. Keeping the plate away from drafts and other temperature fluctuations in the dark.
8. Add 50µl of **Stop Solution** to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the IL-6 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

LIMITATIONS OF THE PROCEDURE

- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- It is important that the Calibrator Diluent selected for the standard curve be consistent with

appropriate Calibrator Diluent and repeat the assay.

- Any variation in Standard Diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

7. TNF-α S U S # . N

- **Plasma**. Collect plasma using citrate, EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 g within 30 minutes of collection. Assay immediately or aliquot and store samples at -20° C. Avoid repeated freeze-thaw cycles.

Note: Grossly hemolyzed samples are not suitable for use in this assay.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and controls be assayed in duplicate.

1. Add 100µl of Standard, Blank, or Sample per well. Cover with the adhesive strip. Incubate for 2 hours at 37° C.
2. Remove the liquid of each well, don't wash.
3. Add 100µl of **Biotin-antibody** working solution to each well. Incubate for 1 hour at 37°C. **Biotin-antibody** working solution may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.
4. Aspirate each well and wash, repeating the process three times for a total of three washes. Wash by filling each well with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

5. Add 100µl of **HRP-avidin** working solution to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37°C.
6. Repeat the aspiration and wash three times as step 4.
7. Add 90µl of **TMB Substrate** to each well. Incubate for 10-30 minutes at 37°C. Keeping the plate away from drafts and other temperature fluctuations in the dark.
8. Add 50µl of **Stop Solution** to each well when the first four wells containing the highest concentration of standards develop obvious blue color. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the TNF-α concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

8. IL-10 ELISA KIT (CUSABIO, CAT#. ER3TNFA)

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.

SAMPLE COLLECTION AND STORAGE

- **Cell Culture Supernates** Remove particulates by centrifugation and assay immediately or aliquot and store samples at -20° C. Avoid repeated freeze-thaw cycles.
- **Serum** Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at -20° C. Avoid repeated freeze-thaw cycles.
- **Plasma** Collect plasma using citrate, EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at -20° C. Avoid repeated freeze-thaw cycles.

Note: Grossly hemolyzed samples are not suitable for use in this assay.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and controls be assayed in duplicate.

1. Add 100µl of Standard, Blank, or Sample per well. Cover with the adhesive strip. Incubate for 2 hours at 37° C.
2. Remove the liquid of each well, don't wash.
3. Add 100µl of **Biotin-antibody** working solution to each well. Incubate for 1 hour at 37° C. **Biotin-antibody** working solution may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.
4. Aspirate each well and wash, repeating the process three times for a total of three washes. Wash by filling each well with Wash Buffer (350µl) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

5. Add 100µl of **HRP-avidin** working solution to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37° C.
6. Repeat the aspiration and wash three times as step 4.
7. Add 90µl of **TMB Substrate** to each well. Incubate for 30 minutes at 37°C. Keeping the plate away from drafts and other temperature fluctuations in the dark.
8. Add 50µl of **Stop Solution** to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

CALCULATION OF RESULTS

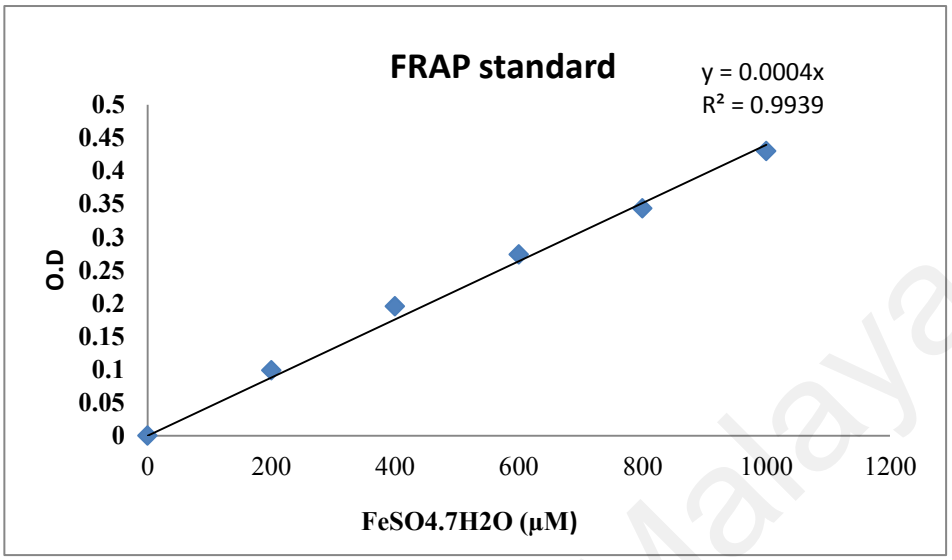
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the IL-10 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

LIMITATIONS OF THE PROCEDURE

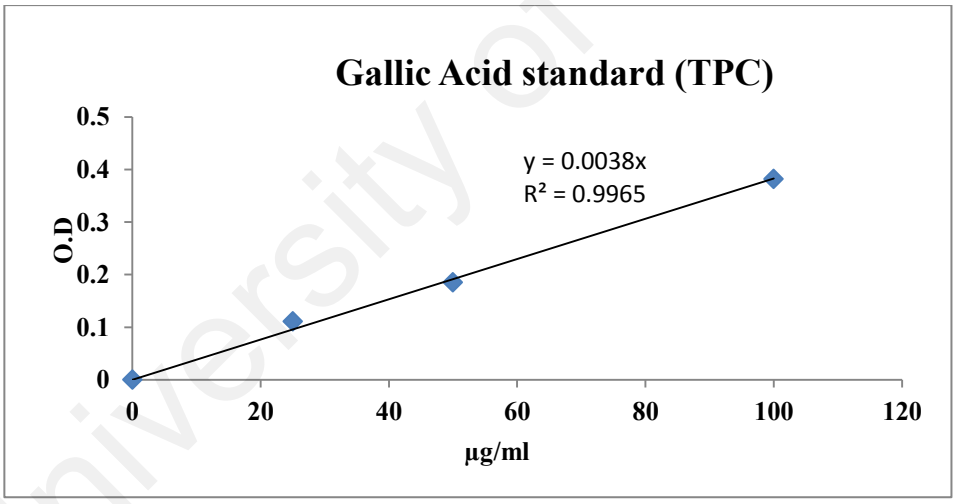
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- It is important that the Calibrator Diluent selected for the standard curve be consistent with the samples being assayed.
- If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- Any variation in Standard Diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

APPENDIX C: PREPARATION OF STANDARD CURVES

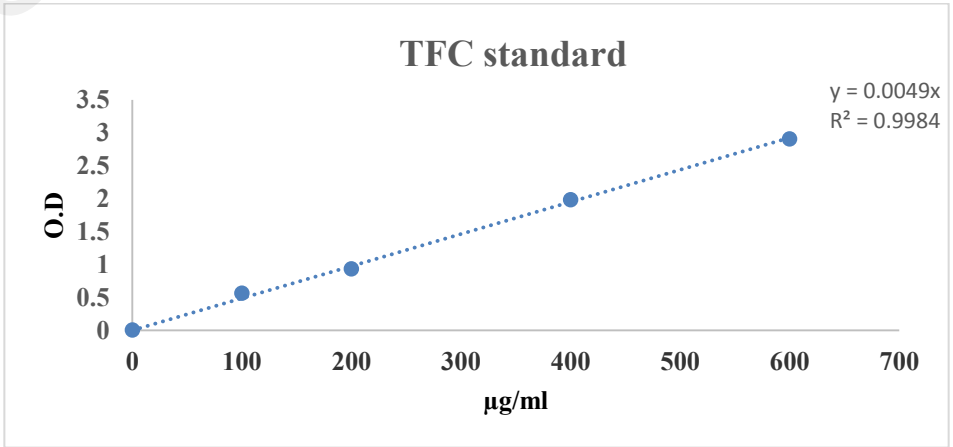
1. FRAP STANDARD CURVE



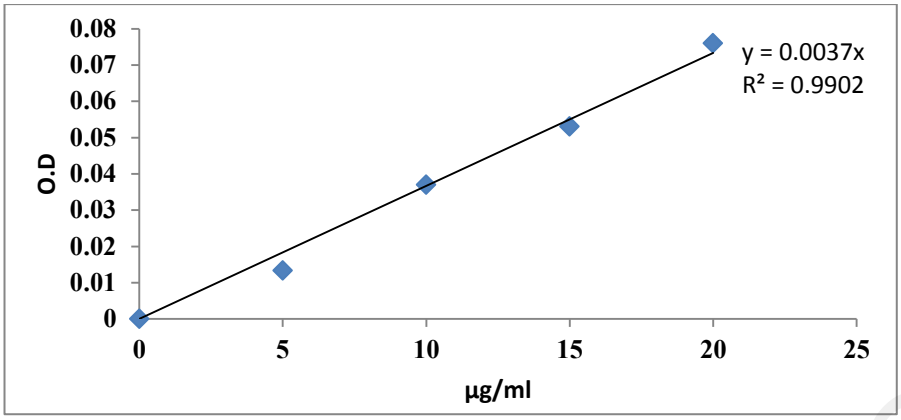
2. GALLIC ACID STANDARD CURVE



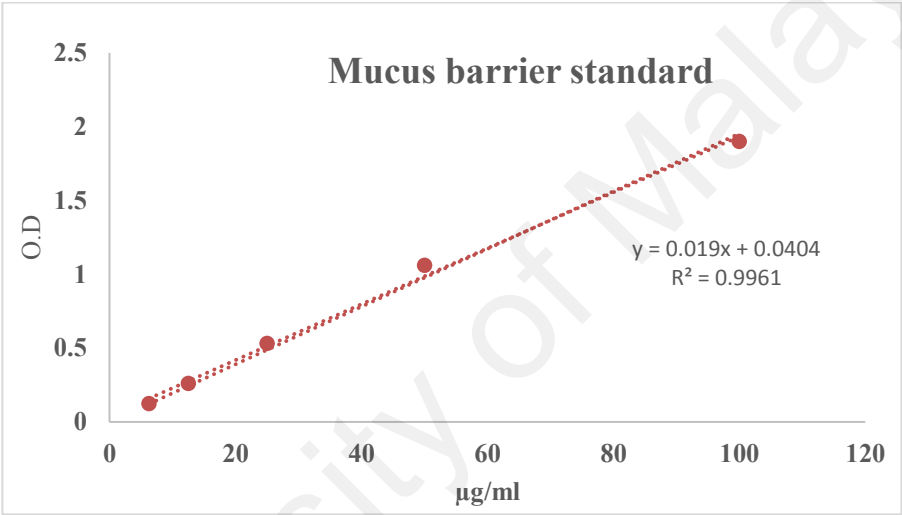
3. TOTAL FLAVANOID CONTENT STANDARD CURVE



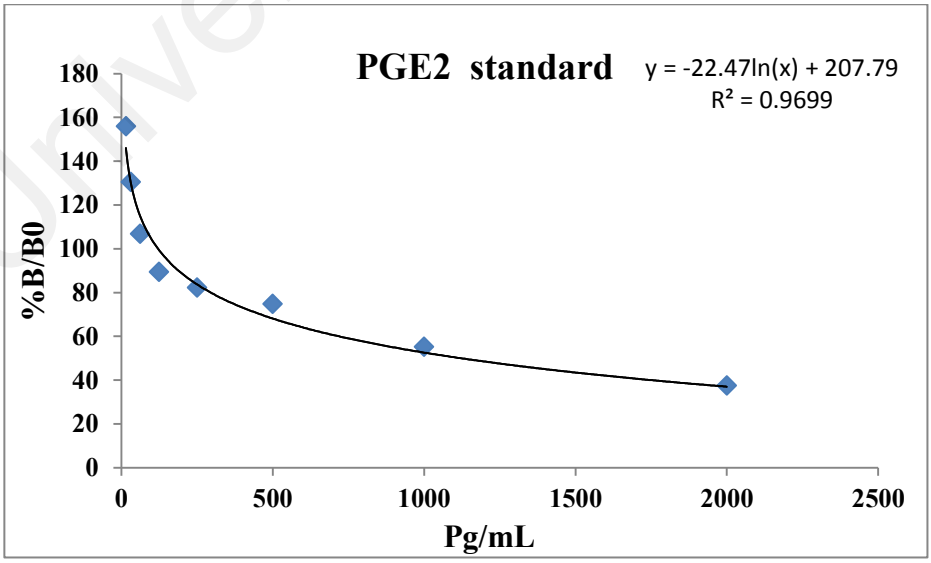
4. BOVIN SERUM ALBUMIN STANDARD CURVE



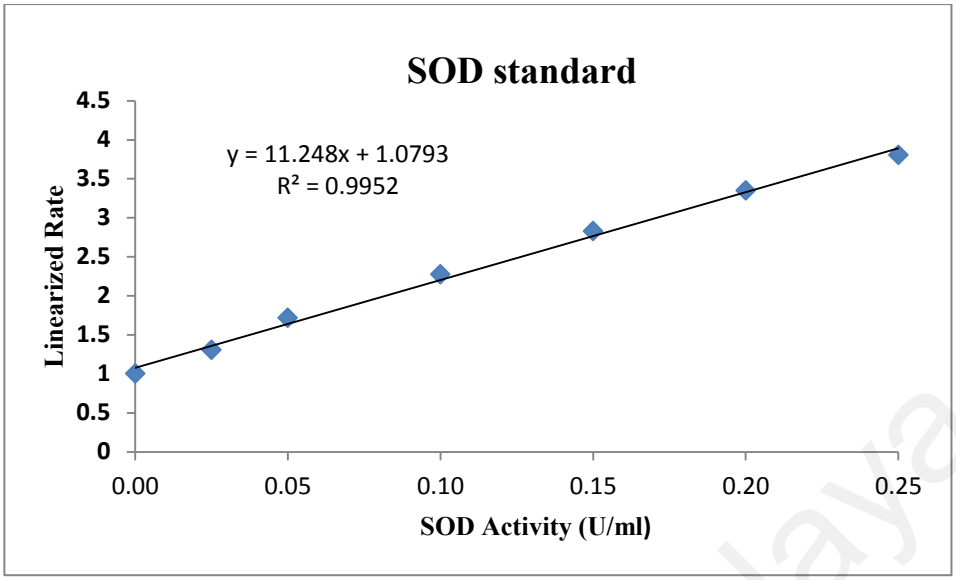
5. ALCIAN BLUE STANDARD CURVE



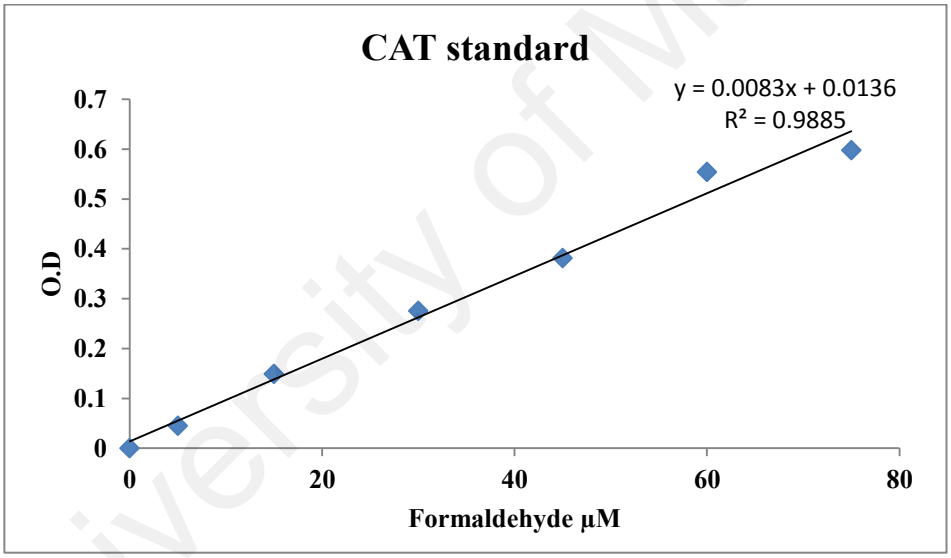
6. PROSTAGLANDIN STANDARD CURVE



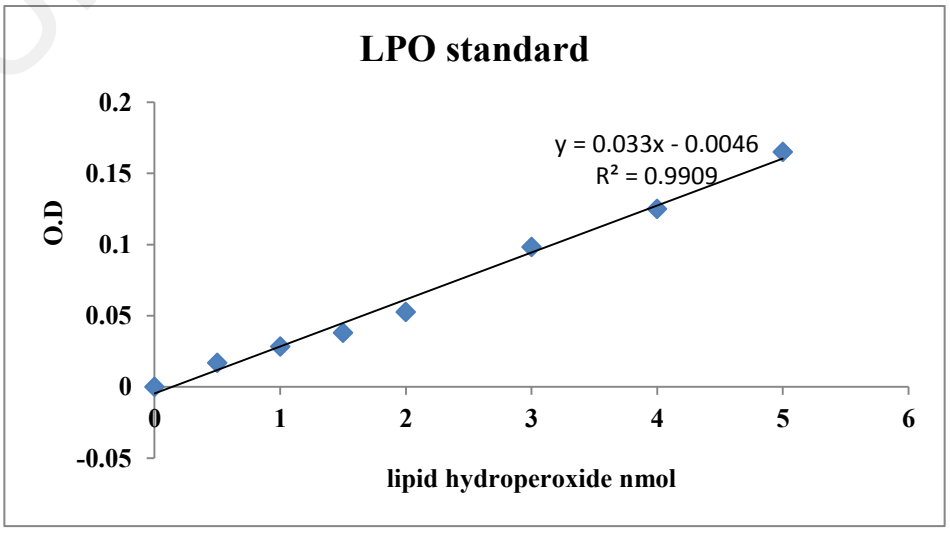
7. SUPEROXIDE DISMUTASE STANDARD CURVE



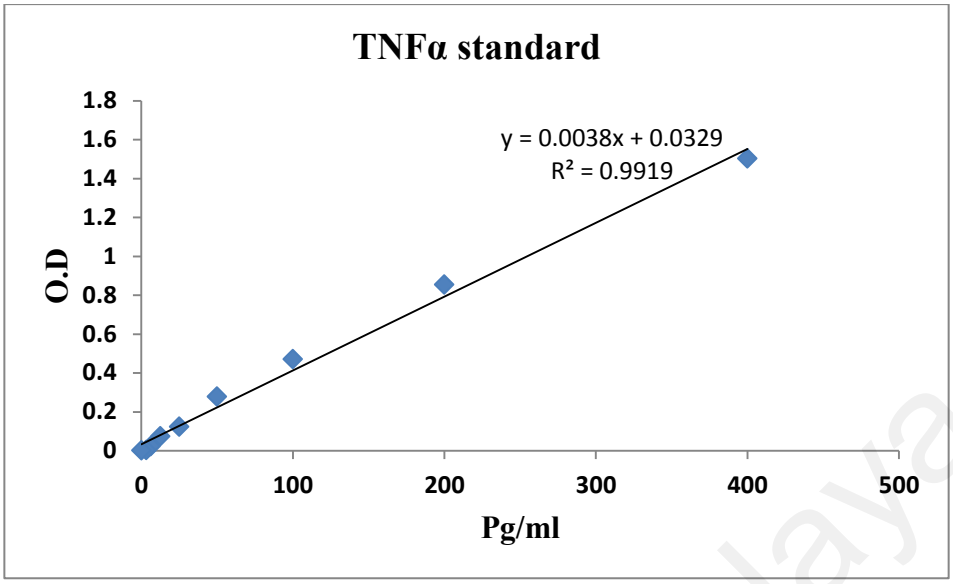
8. CATALASE STANDARD CURVE



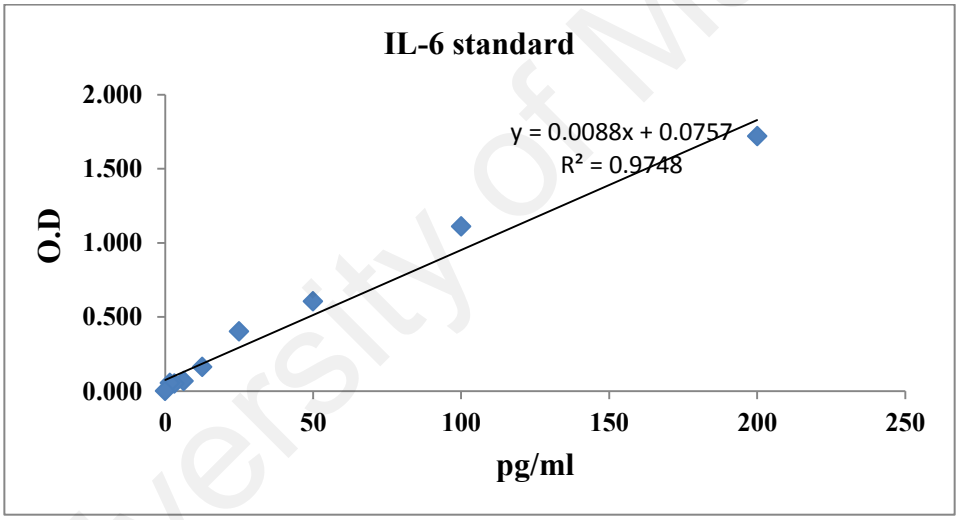
9. LIPID PEROXIDATION STANDARD CURVE



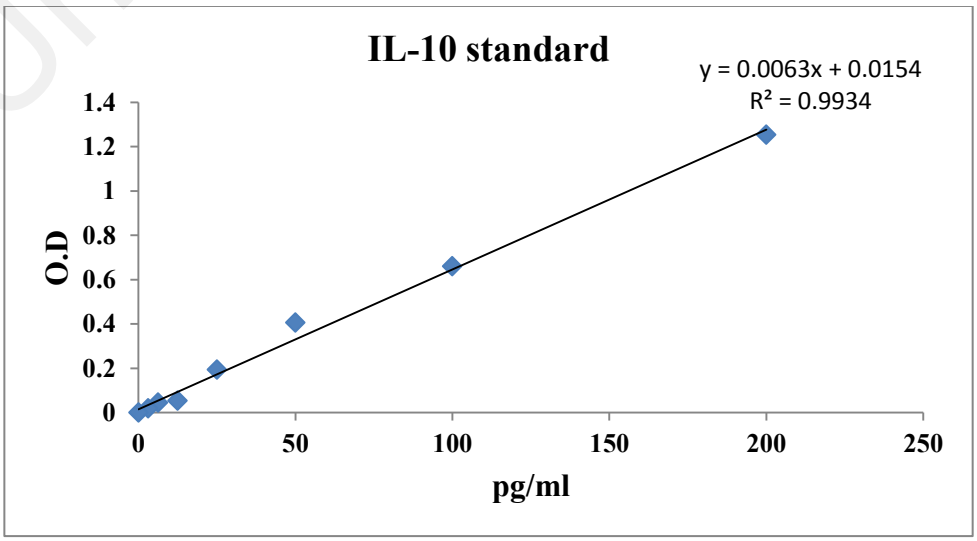
10. TUMOR NECROSIS FACTOR STANDARD CURVE



11. INTERLEUKINE_6 STANDARD CURVE



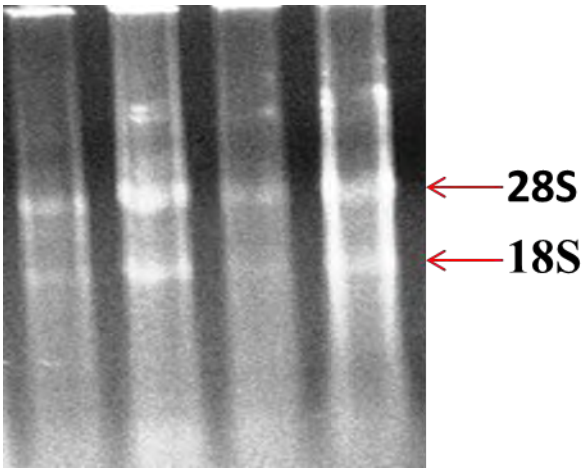
12. INTERLEUKINE_10 STANDARD CURVE



RNA concentration and purity

Sample	**RNA ng/µL**	A260/280
Control 1	917.6	2.13
Control 2	549.1	2.07
Control 3	1334	2.02
Control 4	300.1	1.9
Control 5	403.84	2.01
Reference drug 1	661.64	2.05
Reference drug 2	124.7	2.02
Reference drug 3	424.32	1.71
Reference drug 4	199.2	2.13
Reference drug 5	273.9	2
C.E1 (200 mg/mL)	329.52	2.01
C.E2 (200 mg/mL)	297	2.03
C.E3 (200 mg/mL)	358.1	2.1
C.E4 (200 mg/mL)	167.5	2.09
C.E5 (200 mg/mL)	573.3	2.01

Measurement by Nano Drop ND-2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).



mRNA integrity profile ethidium bromide stained with agarose gel

APPENDIX D: STATISTICAL ANALYSIS

Multiple Comparisons

1. Wound contraction

Dependent Variable	(I) VAR00029	(J) VAR00029	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
five	Gum acacia	Drug	.99394	6.33216	1.000	-17.9543	19.9421
		HD	-13.55943	6.33216	.242	-32.5076	5.3888
		MD	-13.26219	6.33216	.261	-32.2104	5.6860
		LD	-17.57943	6.33216	.077	-36.5276	1.3688
	Drug	Gum acacia	-.99394	6.33216	1.000	-19.9421	17.9543
		HD	-14.55337	6.33216	.186	-33.5016	4.3948
		MD	-14.25613	6.33216	.202	-33.2043	4.6921
		LD	-18.57337	6.33216	.056	-37.5216	.3748
	HD	Gum acacia	13.55943	6.33216	.242	-5.3888	32.5076
		Drug	14.55337	6.33216	.186	-4.3948	33.5016
		MD	.29724	6.33216	1.000	-18.6510	19.2454
		LD	-4.02000	6.33216	.967	-22.9682	14.9282
	MD	Gum acacia	13.26219	6.33216	.261	-5.6860	32.2104
		Drug	14.25613	6.33216	.202	-4.6921	33.2043
		HD	-.29724	6.33216	1.000	-19.2454	18.6510
		LD	-4.31724	6.33216	.958	-23.2654	14.6310
	LD	Gum acacia	17.57943	6.33216	.077	-1.3688	36.5276
		Drug	18.57337	6.33216	.056	-.3748	37.5216
		HD	4.02000	6.33216	.967	-14.9282	22.9682
		MD	4.31724	6.33216	.958	-14.6310	23.2654
Ten	Gum acacia	Drug	-11.47797*	3.38140	.021	-21.5964	-1.3596
		HD	-15.70460*	3.38140	.001	-25.8230	-5.5862
		MD	-16.91902*	3.38140	.001	-27.0374	-6.8006
		LD	-19.50523*	3.38140	.000	-29.6236	-9.3868
	Drug	Gum acacia	11.47797*	3.38140	.021	1.3596	21.5964
		HD	-4.22663	3.38140	.723	-14.3450	5.8918
		MD	-5.44105	3.38140	.509	-15.5595	4.6774
		LD	-8.02725	3.38140	.164	-18.1457	2.0912

		Gum acacia	15.70460*	3.38140	.001	5.5862	25.8230
		Drug	4.22663	3.38140	.723	-5.8918	14.3450
	HD	MD	-1.21442	3.38140	.996	-11.3328	8.9040
		LD	-3.80063	3.38140	.792	-13.9190	6.3178
		Gum acacia	16.91902*	3.38140	.001	6.8006	27.0374
		Drug	5.44105	3.38140	.509	-4.6774	15.5595
	MD	HD	1.21442	3.38140	.996	-8.9040	11.3328
		LD	-2.58621	3.38140	.938	-12.7046	7.5322
		Gum acacia	19.50523*	3.38140	.000	9.3868	29.6236
		Drug	8.02725	3.38140	.164	-2.0912	18.1457
	LD	HD	3.80063	3.38140	.792	-6.3178	13.9190
		MD	2.58621	3.38140	.938	-7.5322	12.7046
		Drug	-16.07014*	2.07302	.000	-22.2734	-9.8669
		HD	-19.92283*	2.07302	.000	-26.1261	-13.7196
	Gum acacia	MD	-18.46977*	2.07302	.000	-24.6730	-12.2665
		LD	-16.79735*	2.07302	.000	-23.0006	-10.5941
		Gum acacia	16.07014*	2.07302	.000	9.8669	22.2734
		HD	-3.85269	2.07302	.370	-10.0559	2.3506
	Drug	MD	-2.39963	2.07302	.774	-8.6029	3.8036
		LD	-.72722	2.07302	.996	-6.9305	5.4760
		Gum acacia	19.92283*	2.07302	.000	13.7196	26.1261
		Drug	3.85269	2.07302	.370	-2.3506	10.0559
Fifteen	HD	MD	1.45306	2.07302	.954	-4.7502	7.6563
		LD	3.12548	2.07302	.570	-3.0778	9.3287
		Gum acacia	18.46977*	2.07302	.000	12.2665	24.6730
		Drug	2.39963	2.07302	.774	-3.8036	8.6029
	MD	HD	-1.45306	2.07302	.954	-7.6563	4.7502
		LD	1.67241	2.07302	.925	-4.5308	7.8757
		Gum acacia	16.79735*	2.07302	.000	10.5941	23.0006
		Drug	.72722	2.07302	.996	-5.4760	6.9305
	LD	HD	-3.12548	2.07302	.570	-9.3287	3.0778
		MD	-1.67241	2.07302	.925	-7.8757	4.5308

*. The mean difference is significant at the 0.05 level.

2. Liver function test statistics

Dependent Variable: AST

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NF	NM	-30.88167	22.78316	.185	-77.4111	15.6478
	HDF	-30.04833	22.78316	.197	-76.5778	16.4811
	LDF	-16.54833	22.78316	.473	-63.0778	29.9811
	HDM	-91.48167*	22.78316	.000	-138.0111	-44.9522
	LDM	-18.24833	22.78316	.429	-64.7778	28.2811
NM	NF	30.88167	22.78316	.185	-15.6478	77.4111
	HDF	.83333	22.78316	.971	-45.6961	47.3628
	LDF	14.33333	22.78316	.534	-32.1961	60.8628
	HDM	-60.60000*	22.78316	.012	-107.1294	-14.0706
	LDM	12.63333	22.78316	.583	-33.8961	59.1628
HDF	NF	30.04833	22.78316	.197	-16.4811	76.5778
	NM	-.83333	22.78316	.971	-47.3628	45.6961
	LDF	13.50000	22.78316	.558	-33.0294	60.0294
	HDM	-61.43333*	22.78316	.011	-107.9628	-14.9039
	LDM	11.80000	22.78316	.608	-34.7294	58.3294
LDF	NF	16.54833	22.78316	.473	-29.9811	63.0778
	NM	-14.33333	22.78316	.534	-60.8628	32.1961
	HDF	-13.50000	22.78316	.558	-60.0294	33.0294
	HDM	-74.93333*	22.78316	.003	-121.4628	-28.4039
	LDM	-1.70000	22.78316	.941	-48.2294	44.8294
HDM	NF	91.48167*	22.78316	.000	44.9522	138.0111
	NM	60.60000*	22.78316	.012	14.0706	107.1294
	HDF	61.43333*	22.78316	.011	14.9039	107.9628
	LDF	74.93333*	22.78316	.003	28.4039	121.4628
	LDM	73.23333*	22.78316	.003	26.7039	119.7628
LDM	NF	18.24833	22.78316	.429	-28.2811	64.7778
	NM	-12.63333	22.78316	.583	-59.1628	33.8961
	HDF	-11.80000	22.78316	.608	-58.3294	34.7294
	LDF	1.70000	22.78316	.941	-44.8294	48.2294
	HDM	-73.23333*	22.78316	.003	-119.7628	-26.7039

*. The mean difference is significant at the 0.05 level.

3. Dependent Variable: Albumin

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NF	NM	2.39333*	.57801	.003	.6352	4.1514
	HDF	-.17333	.57801	1.000	-1.9314	1.5848
	LDF	-.49000	.57801	.956	-2.2481	1.2681
	HDM	.87667	.57801	.657	-.8814	2.6348
	LDM	1.67667	.57801	.068	-.0814	3.4348
NM	NF	-2.39333*	.57801	.003	-4.1514	-.6352
	HDF	-2.56667*	.57801	.001	-4.3248	-.8086
	LDF	-2.88333*	.57801	.000	-4.6414	-1.1252
	HDM	-1.51667	.57801	.122	-3.2748	.2414
	LDM	-.71667	.57801	.814	-2.4748	1.0414
HDF	NF	.17333	.57801	1.000	-1.5848	1.9314
	NM	2.56667*	.57801	.001	.8086	4.3248
	LDF	-.31667	.57801	.994	-2.0748	1.4414
	HDM	1.05000	.57801	.471	-.7081	2.8081
	LDM	1.85000*	.57801	.035	.0919	3.6081
LDF	NF	.49000	.57801	.956	-1.2681	2.2481
	NM	2.88333*	.57801	.000	1.1252	4.6414
	HDF	.31667	.57801	.994	-1.4414	2.0748
	HDM	1.36667	.57801	.201	-.3914	3.1248
	LDM	2.16667*	.57801	.009	.4086	3.9248
HDM	NF	-.87667	.57801	.657	-2.6348	.8814
	NM	1.51667	.57801	.122	-.2414	3.2748
	HDF	-1.05000	.57801	.471	-2.8081	.7081
	LDF	-1.36667	.57801	.201	-3.1248	.3914
	LDM	.80000	.57801	.736	-.9581	2.5581
LDM	NF	-1.67667	.57801	.068	-3.4348	.0814
	NM	.71667	.57801	.814	-1.0414	2.4748
	HDF	-1.85000*	.57801	.035	-3.6081	-.0919
	LDF	-2.16667*	.57801	.009	-3.9248	-.4086
	HDM	-.80000	.57801	.736	-2.5581	.9581

4. Dependent Variable: Bilirubin

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NF	NM	-.26167	.21462	.824	-.9145	.3911
	HDF	-.90500*	.21462	.003	-1.5578	-.2522
	LDF	-.83667*	.21462	.006	-1.4895	-.1839
	HDM	-.49333	.21462	.226	-1.1461	.1595
	LDM	-.81000*	.21462	.008	-1.4628	-.1572
NM	NF	.26167	.21462	.824	-.3911	.9145
	HDF	-.64333	.21462	.055	-1.2961	.0095
	LDF	-.57500	.21462	.109	-1.2278	.0778
	HDM	-.23167	.21462	.886	-.8845	.4211
	LDM	-.54833	.21462	.140	-1.2011	.1045
HDF	NF	.90500*	.21462	.003	.2522	1.5578
	NM	.64333	.21462	.055	-.0095	1.2961
	LDF	.06833	.21462	1.000	-.5845	.7211
	HDM	.41167	.21462	.411	-.2411	1.0645
	LDM	.09500	.21462	.998	-.5578	.7478
LDF	NF	.83667*	.21462	.006	.1839	1.4895
	NM	.57500	.21462	.109	-.0778	1.2278
	HDF	-.06833	.21462	1.000	-.7211	.5845
	HDM	.34333	.21462	.605	-.3095	.9961
	LDM	.02667	.21462	1.000	-.6261	.6795
HDM	NF	.49333	.21462	.226	-.1595	1.1461
	NM	.23167	.21462	.886	-.4211	.8845
	HDF	-.41167	.21462	.411	-1.0645	.2411
	LDF	-.34333	.21462	.605	-.9961	.3095
	LDM	-.31667	.21462	.682	-.9695	.3361
LDM	NF	.81000*	.21462	.008	.1572	1.4628
	NM	.54833	.21462	.140	-.1045	1.2011

	HDF	-.09500	.21462	.998	-.7478	.5578
	LDF	-.02667	.21462	1.000	-.6795	.6261
	HDM	.31667	.21462	.682	-.3361	.9695

*. The mean difference is significant at the 0.05 level.

5. Dependent Variable: ALP

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	NM	-34.61167	24.60293	.170	-84.8575	15.6342
	HDF	-50.25000*	24.60293	.050	-100.4959	-.0041
NF	LDF	33.96667	24.60293	.178	-16.2792	84.2125
	HDM	-105.53333*	24.60293	.000	-155.7792	-55.2875
	LDM	-85.23333*	24.60293	.002	-135.4792	-34.9875
	NF	34.61167	24.60293	.170	-15.6342	84.8575
	HDF	-15.63833	24.60293	.530	-65.8842	34.6075
NM	LDF	68.57833*	24.60293	.009	18.3325	118.8242
	HDM	-70.92167*	24.60293	.007	-121.1675	-20.6758
	LDM	-50.62167*	24.60293	.048	-100.8675	-.3758
	NF	50.25000*	24.60293	.050	.0041	100.4959
	NM	15.63833	24.60293	.530	-34.6075	65.8842
HDF	LDF	84.21667*	24.60293	.002	33.9708	134.4625
	HDM	-55.28333*	24.60293	.032	-105.5292	-5.0375
	LDM	-34.98333	24.60293	.165	-85.2292	15.2625
	NF	-33.96667	24.60293	.178	-84.2125	16.2792
	NM	-68.57833*	24.60293	.009	-118.8242	-18.3325
LDF	HDF	-84.21667*	24.60293	.002	-134.4625	-33.9708
	HDM	-139.50000*	24.60293	.000	-189.7459	-89.2541
	LDM	-119.20000*	24.60293	.000	-169.4459	-68.9541
	NF	105.53333*	24.60293	.000	55.2875	155.7792
	NM	70.92167*	24.60293	.007	20.6758	121.1675
HDM	HDF	55.28333*	24.60293	.032	5.0375	105.5292
	LDF	139.50000*	24.60293	.000	89.2541	189.7459
	LDM	20.30000	24.60293	.416	-29.9459	70.5459
	NF	85.23333*	24.60293	.002	34.9875	135.4792
	NM	50.62167*	24.60293	.048	.3758	100.8675
LDM	HDF	34.98333	24.60293	.165	-15.2625	85.2292
	LDF	119.20000*	24.60293	.000	68.9541	169.4459

	HDM	-20.30000	24.60293	.416	-70.5459	29.9459

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

6. Dependent Variable: ALT

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	NM	-10.27167*	4.43032	.027	-19.3196	-1.2237
	HDF	-7.05500	4.43032	.122	-16.1029	1.9929
NF	LDF	-1.02167	4.43032	.819	-10.0696	8.0263
	HDM	-19.35500*	4.43032	.000	-28.4029	-10.3071
	LDM	-12.35500*	4.43032	.009	-21.4029	-3.3071
	NF	10.27167*	4.43032	.027	1.2237	19.3196
	HDF	3.21667	4.43032	.473	-5.8313	12.2646
NM	LDF	9.25000*	4.43032	.045	.2021	18.2979
	HDM	-9.08333*	4.43032	.049	-18.1313	-.0354
	LDM	-2.08333	4.43032	.642	-11.1313	6.9646
	NF	7.05500	4.43032	.122	-1.9929	16.1029
	NM	-3.21667	4.43032	.473	-12.2646	5.8313
HDF	LDF	6.03333	4.43032	.183	-3.0146	15.0813
	HDM	-12.30000*	4.43032	.009	-21.3479	-3.2521
	LDM	-5.30000	4.43032	.241	-14.3479	3.7479
	NF	1.02167	4.43032	.819	-8.0263	10.0696
	NM	-9.25000*	4.43032	.045	-18.2979	-.2021
LDF	HDF	-6.03333	4.43032	.183	-15.0813	3.0146
	HDM	-18.33333*	4.43032	.000	-27.3813	-9.2854
	LDM	-11.33333*	4.43032	.016	-20.3813	-2.2854
	NF	19.35500*	4.43032	.000	10.3071	28.4029
	NM	9.08333*	4.43032	.049	.0354	18.1313
HDM	HDF	12.30000*	4.43032	.009	3.2521	21.3479
	LDF	18.33333*	4.43032	.000	9.2854	27.3813
	LDM	7.00000	4.43032	.125	-2.0479	16.0479
	NF	12.35500*	4.43032	.009	3.3071	21.4029
	NM	2.08333	4.43032	.642	-6.9646	11.1313
LDM	HDF	5.30000	4.43032	.241	-3.7479	14.3479
	LDF	11.33333*	4.43032	.016	2.2854	20.3813
	HDM	-7.00000	4.43032	.125	-16.0479	2.0479

7. Dependent Variable: Urea

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	NM	-.60667	.35896	.549	-1.6985	.4852
	HDF	-1.97167*	.35896	.000	-3.0635	-.8798
NF	LDF	-.04333	.35896	1.000	-1.1352	1.0485
	HDM	-.14333	.35896	.999	-1.2352	.9485
	LDM	1.18167*	.35896	.028	.0898	2.2735
	NF	.60667	.35896	.549	-.4852	1.6985
	HDF	-1.36500*	.35896	.008	-2.4568	-.2732
NM	LDF	.56333	.35896	.624	-.5285	1.6552
	HDM	.46333	.35896	.788	-.6285	1.5552
	LDM	1.78833*	.35896	.000	.6965	2.8802
	NF	1.97167*	.35896	.000	.8798	3.0635
	NM	1.36500*	.35896	.008	.2732	2.4568
HDF	LDF	1.92833*	.35896	.000	.8365	3.0202
	HDM	1.82833*	.35896	.000	.7365	2.9202
	LDM	3.15333*	.35896	.000	2.0615	4.2452
	NF	.04333	.35896	1.000	-1.0485	1.1352
	NM	-.56333	.35896	.624	-1.6552	.5285
LDF	HDF	-1.92833*	.35896	.000	-3.0202	-.8365
	HDM	-.10000	.35896	1.000	-1.1918	.9918
	LDM	1.22500*	.35896	.021	.1332	2.3168
	NF	.14333	.35896	.999	-.9485	1.2352
HDM	NM	-.46333	.35896	.788	-1.5552	.6285
	HDF	-1.82833*	.35896	.000	-2.9202	-.7365

	LDF	.10000	.35896	1.000	-.9918	1.1918
	LDM	1.32500*	.35896	.010	.2332	2.4168
	NF	-1.18167*	.35896	.028	-2.2735	-.0898
	NM	-1.78833*	.35896	.000	-2.8802	-.6965
LDM	HDF	-3.15333*	.35896	.000	-4.2452	-2.0615
	LDF	-1.22500*	.35896	.021	-2.3168	-.1332
	HDM	-1.32500*	.35896	.010	-2.4168	-.2332

*. The mean difference is significant at the 0.05 level.

8. Dependent Variable: Na

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	NM	.13167	.91879	.887	-1.7447	2.0081
	HDF	-1.87667*	.91879	.050	-3.7531	-.0003
NF	LDF	.05667	.91879	.951	-1.8197	1.9331
	HDM	-1.91833*	.91879	.045	-3.7947	-.0419
	LDM	-1.51000	.91879	.111	-3.3864	.3664
	NF	-.13167	.91879	.887	-2.0081	1.7447
	HDF	-2.00833*	.91879	.037	-3.8847	-.1319
NM	LDF	-.07500	.91879	.935	-1.9514	1.8014
	HDM	-2.05000*	.91879	.033	-3.9264	-.1736
	LDM	-1.64167	.91879	.084	-3.5181	.2347
	NF	1.87667*	.91879	.050	.0003	3.7531
	NM	2.00833*	.91879	.037	.1319	3.8847
HDF	LDF	1.93333*	.91879	.044	.0569	3.8097
	HDM	-.04167	.91879	.964	-1.9181	1.8347
	LDM	.36667	.91879	.693	-1.5097	2.2431
	NF	-.05667	.91879	.951	-1.9331	1.8197
	NM	.07500	.91879	.935	-1.8014	1.9514
LDF	HDF	-1.93333*	.91879	.044	-3.8097	-.0569
	HDM	-1.97500*	.91879	.040	-3.8514	-.0986
	LDM	-1.56667	.91879	.099	-3.4431	.3097
	NF	1.91833*	.91879	.045	.0419	3.7947
	NM	2.05000*	.91879	.033	.1736	3.9264
HDM	HDF	.04167	.91879	.964	-1.8347	1.9181
	LDF	1.97500*	.91879	.040	.0986	3.8514
	LDM	.40833	.91879	.660	-1.4681	2.2847
	NF	1.51000	.91879	.111	-.3664	3.3864
	NM	1.64167	.91879	.084	-.2347	3.5181
LDM	HDF	-.36667	.91879	.693	-2.2431	1.5097
	LDF	1.56667	.91879	.099	-.3097	3.4431
	HDM	-.40833	.91879	.660	-2.2847	1.4681

9. Dependent Variable: K

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	NM	-.18667	.29670	.534	-.7926	.4193
	HDF	-.15500	.29670	.605	-.7610	.4510
NF	LDF	.13233	.29670	.659	-.4736	.7383
	HDM	-.22167	.29670	.461	-.8276	.3843
	LDM	.11167	.29670	.709	-.4943	.7176
	NF	.18667	.29670	.534	-.4193	.7926
	HDF	.03167	.29670	.916	-.5743	.6376
NM	LDF	.31900	.29670	.291	-.2870	.9250
	HDM	-.03500	.29670	.907	-.6410	.5710
	LDM	.29833	.29670	.323	-.3076	.9043
	NF	.15500	.29670	.605	-.4510	.7610
	NM	-.03167	.29670	.916	-.6376	.5743
HDF	LDF	.28733	.29670	.341	-.3186	.8933
	HDM	-.06667	.29670	.824	-.6726	.5393
	LDM	.26667	.29670	.376	-.3393	.8726
	NF	-.13233	.29670	.659	-.7383	.4736
	NM	-.31900	.29670	.291	-.9250	.2870
LDF	HDF	-.28733	.29670	.341	-.8933	.3186
	HDM	-.35400	.29670	.242	-.9600	.2520

	LDM	-.02067	.29670	.945	-.6266	.5853
	NF	.22167	.29670	.461	-.3843	.8276
	NM	.03500	.29670	.907	-.5710	.6410
HDM	HDF	.06667	.29670	.824	-.5393	.6726
	LDF	.35400	.29670	.242	-.2520	.9600
	LDM	.33333	.29670	.270	-.2726	.9393
	NF	-.11167	.29670	.709	-.7176	.4943
	NM	-.29833	.29670	.323	-.9043	.3076
LDM	HDF	-.26667	.29670	.376	-.8726	.3393
	LDF	.02067	.29670	.945	-.5853	.6266
	HDM	-.33333	.29670	.270	-.9393	.2726

10. Dependent Variable: Cl

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	NM	-.52167	1.02194	.613	-2.6088	1.5654
	HDF	-2.49833*	1.02194	.021	-4.5854	-.4112
NF	LDF	-.02667	1.02194	.979	-2.1138	2.0604
	HDM	-.66500	1.02194	.520	-2.7521	1.4221
	LDM	.75167	1.02194	.468	-1.3354	2.8388
	NF	.52167	1.02194	.613	-1.5654	2.6088
	HDF	-1.97667	1.02194	.063	-4.0638	.1104
NM	LDF	.49500	1.02194	.632	-1.5921	2.5821
	HDM	-.14333	1.02194	.889	-2.2304	1.9438
	LDM	1.27333	1.02194	.222	-.8138	3.3604
	NF	2.49833*	1.02194	.021	.4112	4.5854
	NM	1.97667	1.02194	.063	-.1104	4.0638
HDF	LDF	2.47167*	1.02194	.022	.3846	4.5588
	HDM	1.83333	1.02194	.083	-.2538	3.9204
	LDM	3.25000*	1.02194	.003	1.1629	5.3371
	NF	.02667	1.02194	.979	-2.0604	2.1138
	NM	-.49500	1.02194	.632	-2.5821	1.5921
LDF	HDF	-2.47167*	1.02194	.022	-4.5588	-.3846
	HDM	-.63833	1.02194	.537	-2.7254	1.4488
	LDM	.77833	1.02194	.452	-1.3088	2.8654
	NF	.66500	1.02194	.520	-1.4221	2.7521
	NM	.14333	1.02194	.889	-1.9438	2.2304
HDM	HDF	-1.83333	1.02194	.083	-3.9204	.2538
	LDF	.63833	1.02194	.537	-1.4488	2.7254
	LDM	1.41667	1.02194	.176	-.6704	3.5038
	NF	-.75167	1.02194	.468	-2.8388	1.3354
	NM	-1.27333	1.02194	.222	-3.3604	.8138
LDM	HDF	-3.25000*	1.02194	.003	-5.3371	-1.1629
	HDM	-1.41667	1.02194	.176	-3.5038	.6704

11. Mucus barrier

(I) VAR00004	(J) VAR00004	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	OMP	-11.66154*	3.88777	.038	-22.7845	-.5385
NC	HD	-23.30256*	3.88777	.000	-34.4256	-12.1796
	LD	-11.87692*	3.88777	.034	-22.9999	-.7539
	NC	11.66154*	3.88777	.038	.5385	22.7845
OMP	HD	-11.64103*	3.88777	.039	-22.7640	-.5180
	LD	-.21538	3.88777	1.000	-11.3384	10.9076
	NC	23.30256*	3.88777	.000	12.1796	34.4256
HD	OMP	11.64103*	3.88777	.039	.5180	22.7640
	LD	11.42564*	3.88777	.043	.3026	22.5486
	NC	11.87692*	3.88777	.034	.7539	22.9999
LD	OMP	.21538	3.88777	1.000	-10.9076	11.3384
	HD	-11.42564*	3.88777	.043	-22.5486	-.3026

12. Dependent Variable: SOD

(I)	(J)	Mean	Std.	Sig.	95% Confidence Interval

VAR00006	VAR00006	Difference (I-J)	Error		Lower Bound	Upper Bound
NC	Drug	-3.07500*	.92945	.022	-5.8047	-.3453
	HD	-2.73000*	.92945	.050	-5.4597	-.0003
	MD	-3.55667*	.92945	.006	-6.2863	-.8270
	LD	-2.15500	.92945	.172	-4.8847	.5747
Drug	NC	3.07500*	.92945	.022	.3453	5.8047
	HD	.34500	.92945	.996	-2.3847	3.0747
	MD	-.48167	.92945	.985	-3.2113	2.2480
	LD	.92000	.92945	.857	-1.8097	3.6497
HD	NC	2.73000*	.92945	.050	.0003	5.4597
	Drug	-.34500	.92945	.996	-3.0747	2.3847
	MD	-.82667	.92945	.898	-3.5563	1.9030
	LD	.57500	.92945	.971	-2.1547	3.3047
MD	NC	3.55667*	.92945	.006	.8270	6.2863
	Drug	.48167	.92945	.985	-2.2480	3.2113
	HD	.82667	.92945	.898	-1.9030	3.5563
	LD	1.40167	.92945	.567	-1.3280	4.1313
LD	NC	2.15500	.92945	.172	-.5747	4.8847
	Drug	-.92000	.92945	.857	-3.6497	1.8097
	HD	-.57500	.92945	.971	-3.3047	2.1547
	MD	-1.40167	.92945	.567	-4.1313	1.3280

13. Dependent Variable: CAT

(I) VAR00011	(J) VAR00011	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
gum acacia	Drug	-9.08600*	3.15901	.009	-15.6756	-2.4964
	HD	-10.72000*	3.15901	.003	-17.3096	-4.1304
	MD	-5.94600	3.15901	.074	-12.5356	.6436
	LD	-2.54800	3.15901	.429	-9.1376	4.0416
Drug	gum acacia	9.08600*	3.15901	.009	2.4964	15.6756
	HD	-1.63400	3.15901	.611	-8.2236	4.9556
	MD	3.14000	3.15901	.332	-3.4496	9.7296
	LD	6.53800	3.15901	.052	-.0516	13.1276
HD	gum acacia	10.72000*	3.15901	.003	4.1304	17.3096
	Drug	1.63400	3.15901	.611	-4.9556	8.2236
	MD	4.77400	3.15901	.146	-1.8156	11.3636
	LD	8.17200*	3.15901	.018	1.5824	14.7616
MD	gum acacia	5.94600	3.15901	.074	-.6436	12.5356
	Drug	-3.14000	3.15901	.332	-9.7296	3.4496
	HD	-4.77400	3.15901	.146	-11.3636	1.8156
	LD	3.39800	3.15901	.295	-3.1916	9.9876
LD	gum acacia	2.54800	3.15901	.429	-4.0416	9.1376
	Drug	-6.53800	3.15901	.052	-13.1276	.0516
	HD	-8.17200*	3.15901	.018	-14.7616	-1.5824
	MD	-3.39800	3.15901	.295	-9.9876	3.1916

14. 1Dependent Variable: GPX

(I) VAR00021	(J) VAR00021	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Gum acacia	Drug	-16.59167*	2.55878	.000	-21.8616	-11.3218
	HD	-6.28167*	2.55878	.021	-11.5516	-1.0118
	MD	-13.19667*	2.55878	.000	-18.4666	-7.9268
	LD	-5.12500	2.55878	.056	-10.3949	.1449
Drug	Gum acacia	16.59167*	2.55878	.000	11.3218	21.8616
	HD	10.31000*	2.55878	.000	5.0401	15.5799
	MD	3.39500	2.55878	.197	-1.8749	8.6649
	LD	11.46667*	2.55878	.000	6.1968	16.7366
HD	Gum acacia	6.28167*	2.55878	.021	1.0118	11.5516
	Drug	-10.31000*	2.55878	.000	-15.5799	-5.0401
	MD	-6.91500*	2.55878	.012	-12.1849	-1.6451
	LD	1.15667	2.55878	.655	-4.1132	6.4266
MD	Gum acacia	13.19667*	2.55878	.000	7.9268	18.4666
	Drug	-3.39500	2.55878	.197	-8.6649	1.8749

	HD	6.91500*	2.55878	.012	1.6451	12.1849
	LD	8.07167*	2.55878	.004	2.8018	13.3416
	Gum acacia	5.12500	2.55878	.056	-.1449	10.3949
LD	Drug	-11.46667*	2.55878	.000	-16.7366	-6.1968
	HD	-1.15667	2.55878	.655	-6.4266	4.1132
	MD	-8.07167*	2.55878	.004	-13.3416	-2.8018

15. Dependent Variable: LPO

(I) VAR00016	(J) VAR00016	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Gum acacia	Drug	6.31000*	1.59752	.001	2.9776	9.6424
	HD	4.95200*	1.59752	.006	1.6196	8.2844
	MD	4.27400*	1.59752	.015	.9416	7.6064
	LD	5.15600*	1.59752	.004	1.8236	8.4884
Drug	Gum acacia	-6.31000*	1.59752	.001	-9.6424	-2.9776
	HD	-1.35800	1.59752	.405	-4.6904	1.9744
	MD	-2.03600	1.59752	.217	-5.3684	1.2964
	LD	-1.15400	1.59752	.478	-4.4864	2.1784
HD	Gum acacia	-4.95200*	1.59752	.006	-8.2844	-1.6196
	Drug	1.35800	1.59752	.405	-1.9744	4.6904
	MD	-.67800	1.59752	.676	-4.0104	2.6544
	LD	.20400	1.59752	.900	-3.1284	3.5364
MD	Gum acacia	-4.27400*	1.59752	.015	-7.6064	-.9416
	Drug	2.03600	1.59752	.217	-1.2964	5.3684
	HD	.67800	1.59752	.676	-2.6544	4.0104
	LD	.88200	1.59752	.587	-2.4504	4.2144
LD	Gum acacia	-5.15600*	1.59752	.004	-8.4884	-1.8236
	Drug	1.15400	1.59752	.478	-2.1784	4.4864
	HD	-.20400	1.59752	.900	-3.5364	3.1284
	MD	-.88200	1.59752	.587	-4.2144	2.4504

*. The mean difference is significant at the 0.05 level.

16. Dependent Variable: IL6

(I) VAR00029	(J) VAR00029	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Gum acacia	Drug	96.08693*	20.81248	.000	52.6729	139.5010
	HD	106.83011*	20.81248	.000	63.4160	150.2442
	MD	96.64148*	20.81248	.000	53.2274	140.0556
	LD	103.38693*	20.81248	.000	59.9729	146.8010
Drug	Gum acacia	-96.08693*	20.81248	.000	-139.5010	-52.6729
	HD	10.74318	20.81248	.611	-32.6709	54.1573
	MD	.55455	20.81248	.979	-42.8595	43.9686
	LD	7.30000	20.81248	.729	-36.1141	50.7141
HD	Gum acacia	-106.83011*	20.81248	.000	-150.2442	-63.4160
	Drug	-10.74318	20.81248	.611	-54.1573	32.6709
	MD	-10.18864	20.81248	.630	-53.6027	33.2254
	LD	-3.44318	20.81248	.870	-46.8573	39.9709
MD	Gum acacia	-96.64148*	20.81248	.000	-140.0556	-53.2274
	Drug	-.55455	20.81248	.979	-43.9686	42.8595

	HD	10.18864	20.81248	.630	-33.2254	53.6027
	LD	6.74545	20.81248	.749	-36.6686	50.1595
	Gum acacia	-103.38693*	20.81248	.000	-146.8010	-59.9729
	Drug	-7.30000	20.81248	.729	-50.7141	36.1141
LD	HD	3.44318	20.81248	.870	-39.9709	46.8573
	MD	-6.74545	20.81248	.749	-50.1595	36.6686

17. Dependent Variable: TNF

(I) VAR00029	(J) VAR00029	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	Drug	17.11184	10.94609	.134	-5.7213	39.9450
	HD	33.26974*	10.94609	.006	10.4366	56.1029
Gum acacia	MD	41.24342*	10.94609	.001	18.4103	64.0766
	LD	19.97105	10.94609	.083	-2.8621	42.8042
	Gum acacia	-17.11184	10.94609	.134	-39.9450	5.7213
	HD	16.15789	10.94609	.155	-6.6752	38.9910
Drug	MD	24.13158*	10.94609	.039	1.2984	46.9647
	LD	2.85921	10.94609	.797	-19.9739	25.6923
	Gum acacia	-33.26974*	10.94609	.006	-56.1029	-10.4366
	Drug	-16.15789	10.94609	.155	-38.9910	6.6752
HD	MD	7.97368	10.94609	.475	-14.8595	30.8068
	LD	-13.29868	10.94609	.239	-36.1318	9.5345
	Gum acacia	-41.24342*	10.94609	.001	-64.0766	-18.4103
	Drug	-24.13158*	10.94609	.039	-46.9647	-1.2984
MD	HD	-7.97368	10.94609	.475	-30.8068	14.8595
	LD	-21.27237	10.94609	.066	-44.1055	1.5608
	Gum acacia	-19.97105	10.94609	.083	-42.8042	2.8621
	Drug	-2.85921	10.94609	.797	-25.6923	19.9739
LD	HD	13.29868	10.94609	.239	-9.5345	36.1318
	MD	21.27237	10.94609	.066	-1.5608	44.1055

18. Dependent Variable: IL10

(I) VAR00029	(J) VAR00029	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	Drug	-17.67000	17.71875	.331	-54.6307	19.2907
Gum acacia	HD	-54.33750*	17.71875	.006	-91.2982	-17.3768
	MD	-30.91000	17.71875	.096	-67.8707	6.0507

Drug	LD	-7.71250	17.7187 5	.668	-44.6732	29.2482
	Gum acacia	17.67000	17.7187 5	.331	-19.2907	54.6307
	HD	-36.66750	17.7187 5	.052	-73.6282	.2932
	MD	-13.24000	17.7187 5	.464	-50.2007	23.7207
HD	LD	9.95750	17.7187 5	.580	-27.0032	46.9182
	Gum acacia	54.33750*	17.7187 5	.006	17.3768	91.2982
	Drug	36.66750	17.7187 5	.052	-.2932	73.6282
	MD	23.42750	17.7187 5	.201	-13.5332	60.3882
MD	LD	46.62500*	17.7187 5	.016	9.6643	83.5857
	Gum acacia	30.91000	17.7187 5	.096	-6.0507	67.8707
	Drug	13.24000	17.7187 5	.464	-23.7207	50.2007
	HD	-23.42750	17.7187 5	.201	-60.3882	13.5332
LD	LD	23.19750	17.7187 5	.205	-13.7632	60.1582
	Gum acacia	7.71250	17.7187 5	.668	-29.2482	44.6732
	Drug	-9.95750	17.7187 5	.580	-46.9182	27.0032
	HD	-46.62500*	17.7187 5	.016	-83.5857	-9.6643
	MD	-23.19750	17.7187 5	.205	-60.1582	13.7632

*. The mean difference is significant at the 0.05 level.

19. Dependent Variable: SOD

(I) VAR00009	(J) VAR00009	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NC	UC	.24287	.16217	.147	-.0911	.5769
	OMP	-.15355	.16217	.353	-.4875	.1804
	HD	-.26805	.16217	.111	-.6020	.0659
	LD	-.23465	.16217	.160	-.5686	.0993
UC	NC	-.24287	.16217	.147	-.5769	.0911
	OMP	-.39642*	.16217	.022	-.7304	-.0624
	HD	-.51092*	.16217	.004	-.8449	-.1769
	LD	-.47751*	.16217	.007	-.8115	-.1435
OMP	NC	.15355	.16217	.353	-.1804	.4875
	UC	.39642*	.16217	.022	.0624	.7304
	HD	-.11450	.16217	.487	-.4485	.2195
	LD	-.08110	.16217	.621	-.4151	.2529
HD	NC	.26805	.16217	.111	-.0659	.6020
	UC	.51092*	.16217	.004	.1769	.8449
	OMP	.11450	.16217	.487	-.2195	.4485
	LD	.03340	.16217	.838	-.3006	.3674
LD	NC	.23465	.16217	.160	-.0993	.5686
	UC	.47751*	.16217	.007	.1435	.8115
	OMP	.08110	.16217	.621	-.2529	.4151
	HD	-.03340	.16217	.838	-.3674	.3006

20. Dependent Variable: CAT

(I) VAR00012	(J) VAR00012	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NC	UC	43.20407*	6.26286	.000	30.1400	56.2682
	OMP	14.35271*	6.26286	.033	1.2886	27.4168

	HD	-32.73780*	6.26286	.000	-45.8019	-19.6737
	LD	-9.30520	6.26286	.153	-22.3693	3.7589
	NC	-43.20407*	6.26286	.000	-56.2682	-30.1400
UC	OMP	-28.85136*	6.26286	.000	-41.9154	-15.7873
	HD	-75.94187*	6.26286	.000	-89.0060	-62.8778
	LD	-52.50926*	6.26286	.000	-65.5734	-39.4452
	NC	-14.35271*	6.26286	.033	-27.4168	-1.2886
OMP	UC	28.85136*	6.26286	.000	15.7873	41.9154
	HD	-47.09051*	6.26286	.000	-60.1546	-34.0264
	LD	-23.65791*	6.26286	.001	-36.7220	-10.5938
	NC	32.73780*	6.26286	.000	19.6737	45.8019
HD	UC	75.94187*	6.26286	.000	62.8778	89.0060
	OMP	47.09051*	6.26286	.000	34.0264	60.1546
	LD	23.43261*	6.26286	.001	10.3685	36.4967
	NC	9.30520	6.26286	.153	-3.7589	22.3693
LD	UC	52.50926*	6.26286	.000	39.4452	65.5734
	OMP	23.65791*	6.26286	.001	10.5938	36.7220
	HD	-23.43261*	6.26286	.001	-36.4967	-10.3685

*. The mean difference is significant at the 0.05 level.

21. Dependent Variable: GPx

(I) VAR00018	(J) VAR00018	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	UC	22.26667*	6.44629	.002	8.9903	35.5431
	OMP	-1.98333	6.44629	.761	-15.2597	11.2931
NC	HD	1.16667	6.44629	.858	-12.1097	14.4431
	LD	12.46667	6.44629	.065	-.8097	25.7431
	NC	-22.26667*	6.44629	.002	-35.5431	-8.9903
	OMP	-24.25000*	6.44629	.001	-37.5264	-10.9736
UC	HD	-21.10000*	6.44629	.003	-34.3764	-7.8236
	LD	-9.80000	6.44629	.141	-23.0764	3.4764
	NC	1.98333	6.44629	.761	-11.2931	15.2597
	UC	24.25000*	6.44629	.001	10.9736	37.5264
OMP	HD	3.15000	6.44629	.629	-10.1264	16.4264
	LD	14.45000*	6.44629	.034	1.1736	27.7264
	NC	-1.16667	6.44629	.858	-14.4431	12.1097
	UC	21.10000*	6.44629	.003	7.8236	34.3764
HD	OMP	-3.15000	6.44629	.629	-16.4264	10.1264
	LD	11.30000	6.44629	.092	-1.9764	24.5764
	NC	-12.46667	6.44629	.065	-25.7431	.8097
	UC	9.80000	6.44629	.141	-3.4764	23.0764
LD	OMP	-14.45000*	6.44629	.034	-27.7264	-1.1736
	HD	-11.30000	6.44629	.092	-24.5764	1.9764

*. The mean difference is significant at the 0.05 level.

22. Dependent Variable: LPO

(I) VAR00014	(J) VAR00014	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	UC	-7.01212*	1.48617	.000	-10.0729	-3.9513
	OMP	-2.05758	1.48617	.178	-5.1184	1.0032
NC	HD	-2.63030	1.48617	.089	-5.6911	.4305
	LD	-1.71818	1.48617	.259	-4.7790	1.3426
	NC	7.01212*	1.48617	.000	3.9513	10.0729
	OMP	4.95455*	1.48617	.003	1.8937	8.0154
UC	HD	4.38182*	1.48617	.007	1.3210	7.4426
	LD	5.29394*	1.48617	.002	2.2331	8.3548
	NC	2.05758	1.48617	.178	-1.0032	5.1184
	UC	-4.95455*	1.48617	.003	-8.0154	-1.8937
OMP	HD	-.57273	1.48617	.703	-3.6336	2.4881
	LD	.33939	1.48617	.821	-2.7214	3.4002
	NC	2.63030	1.48617	.089	-.4305	5.6911
	UC	-4.38182*	1.48617	.007	-7.4426	-1.3210
HD	OMP	.57273	1.48617	.703	-2.4881	3.6336
	LD	.91212	1.48617	.545	-2.1487	3.9729
LD	NC	1.71818	1.48617	.259	-1.3426	4.7790

	UC	-5.29394*	1.48617	.002	-8.3548	-2.2331
	OMP	-.33939	1.48617	.821	-3.4002	2.7214
	HD	-.91212	1.48617	.545	-3.9729	2.1487

23. Dependent Variable: PGE2

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NC	UC	2.90873*	.47648	.000	1.5094	4.3081
	OMP	.61941	.47648	.694	-.7800	2.0188
	HD	.66810	.47648	.632	-.7313	2.0675
	LD	1.20183	.47648	.118	-.1975	2.6012
UC	NC	-2.90873*	.47648	.000	-4.3081	-1.5094
	OMP	-2.28932*	.47648	.001	-3.6887	-.8900
	HD	-2.24064*	.47648	.001	-3.6400	-.8413
	LD	-1.70690*	.47648	.011	-3.1063	-.3075
OMP	NC	-.61941	.47648	.694	-2.0188	.7800
	UC	2.28932*	.47648	.001	.8900	3.6887
	HD	.04868	.47648	1.000	-1.3507	1.4480
	LD	.58242	.47648	.739	-.8169	1.9818
HD	NC	-.66810	.47648	.632	-2.0675	.7313
	UC	2.24064*	.47648	.001	.8413	3.6400
	OMP	-.04868	.47648	1.000	-1.4480	1.3507
	LD	.53374	.47648	.794	-.8656	1.9331
LD	NC	-1.20183	.47648	.118	-2.6012	.1975
	UC	1.70690*	.47648	.011	.3075	3.1063
	OMP	-.58242	.47648	.739	-1.9818	.8169
	HD	-.53374	.47648	.794	-1.9331	.8656

24. Dependent Variable: VAR00027 TNF

(I) VAR00024	(J) VAR00024	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NC	UC	-97.34000*	11.27206	.000	-120.8531	-73.8269
	OMP	-44.26632*	11.27206	.001	-67.7794	-20.7532
	HD	-50.02684*	11.27206	.000	-73.5399	-26.5137
	LD	-54.38000*	11.27206	.000	-77.8931	-30.8669
UC	NC	97.34000*	11.27206	.000	73.8269	120.8531
	OMP	53.07368*	11.27206	.000	29.5606	76.5868
	HD	47.31316*	11.27206	.000	23.8001	70.8263
	LD	42.96000*	11.27206	.001	19.4469	66.4731
OMP	NC	44.26632*	11.27206	.001	20.7532	67.7794
	UC	-53.07368*	11.27206	.000	-76.5868	-29.5606
	HD	-5.76053	11.27206	.615	-29.2736	17.7526
	LD	-10.11368	11.27206	.380	-33.6268	13.3994
HD	NC	50.02684*	11.27206	.000	26.5137	73.5399
	UC	-47.31316*	11.27206	.000	-70.8263	-23.8001
	OMP	5.76053	11.27206	.615	-17.7526	29.2736
	LD	-4.35316	11.27206	.703	-27.8663	19.1599
LD	NC	54.38000*	11.27206	.000	30.8669	77.8931

	UC	-42.96000*	11.27206	.001	-66.4731	-19.4469
	OMP	10.11368	11.27206	.380	-13.3994	33.6268
	HD	4.35316	11.27206	.703	-19.1599	27.8663

*. The mean difference is significant at the 0.05 level.

25. Dependent Variable: IL6

(I) VAR00024	(J) VAR00024	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NC	UC	-91.00000*	16.956778	.000	-126.3712	-55.6288
	OMP	-47.94886*	16.956778	.010	-83.3201	-12.5776
	HD	-12.65625	16.956778	.464	-48.0275	22.7150
	LD	-13.72443	16.956778	.428	-49.0957	21.6468
UC	NC	91.00000*	16.956778	.000	55.6288	126.3712
	OMP	43.05114*	16.956778	.020	7.6799	78.4224
	HD	78.34375*	16.956778	.000	42.9725	113.7150
	LD	77.27557*	16.956778	.000	41.9043	112.6468
OMP	NC	47.94886*	16.956778	.010	12.5776	83.3201
	UC	-43.05114*	16.956778	.020	-78.4224	-7.6799
	HD	35.29261	16.956778	.050	-.0786	70.6638
	LD	34.22443	16.956778	.057	-1.1468	69.5957
HD	NC	12.65625	16.956778	.464	-22.7150	48.0275
	UC	-78.34375*	16.956778	.000	-113.7150	-42.9725
	OMP	-35.29261	16.956778	.050	-70.6638	.0786
	LD	-1.06818	16.956778	.950	-36.4394	34.3030
LD	NC	13.72443	16.956778	.428	-21.6468	49.0957
	UC	-77.27557*	16.956778	.000	-112.6468	-41.9043
	OMP	-34.22443	16.956778	.057	-69.5957	1.1468
	HD	1.06818	16.956778	.950	-34.3030	36.4394

*. The mean difference is significant at the 0.05 level.

26. Dependent Variable: IL10

(I) VAR00024	(J) VAR00024	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
NC	UC	64.72250*	17.644888	.002	27.9159	101.5291
	OMP	43.06349*	17.644888	.024	6.2569	79.8701
	HD	12.09250	17.644888	.501	-24.7141	48.8991
	LD	4.24798	17.644888	.812	-32.5586	41.0546
UC	NC	-64.72250*	17.644888	.002	-101.5291	-27.9159

	OMP	-21.65901	17.64488	.234	-58.4656	15.1476
	HD	-52.63000*	17.64488	.007	-89.4366	-15.8234
	LD	-60.47452*	17.64488	.003	-97.2811	-23.6679
	NC	-43.06349*	17.64488	.024	-79.8701	-6.2569
OMP	UC	21.65901	17.64488	.234	-15.1476	58.4656
	HD	-30.97099	17.64488	.095	-67.7776	5.8356
	LD	-38.81552*	17.64488	.040	-75.6221	-2.0089
	NC	-12.09250	17.64488	.501	-48.8991	24.7141
	UC	52.63000*	17.64488	.007	15.8234	89.4366
HD	OMP	30.97099	17.64488	.095	-5.8356	67.7776
	LD	-7.84452	17.64488	.661	-44.6511	28.9621
	NC	-4.24798	17.64488	.812	-41.0546	32.5586
	UC	60.47452*	17.64488	.003	23.6679	97.2811
LD	OMP	38.81552*	17.64488	.040	2.0089	75.6221
	HD	7.84452	17.64488	.661	-28.9621	44.6511

Histopathological evaluation of the epidermis and dermis

Number of families	5
Number of comparisons per family	10
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Epithelialization (E)				
Control vs. MFCE-LD	-3.500	-4.475 to -2.525	Yes	****
Control vs. MFCE-MD	-3.500	-4.475 to -2.525	Yes	****
Control vs. MFCE-HD	-3.500	-4.475 to -2.525	Yes	****
Control vs. INTRASITE	-3.500	-4.475 to -2.525	Yes	****
MFCE-LD vs. MFCE-MD	0.0	-0.9747 to 0.9747	No	ns
MFCE-LD vs. MFCE-HD	0.0	-0.9747 to 0.9747	No	ns
MFCE-LD vs. INTRASITE	0.0	-0.9747 to 0.9747	No	ns
MFCE-MD vs. MFCE-HD	0.0	-0.9747 to 0.9747	No	ns
MFCE-MD vs. INTRASITE	0.0	-0.9747 to 0.9747	No	ns
MFCE-HD vs. INTRASITE	0.0	-0.9747 to 0.9747	No	ns
Inflammatory Cell (E)				
Control vs. MFCE-LD	2.200	1.225 to 3.175	Yes	****
Control vs. MFCE-MD	0.8000	-0.1747 to 1.775	No	ns
Control vs. MFCE-HD	2.000	1.025 to 2.975	Yes	****
Control vs. INTRASITE	1.000	0.02526 to	Yes	*

		1.975		
MFCE-LD vs. MFCE-MD	-1.400	-2.375 to -0.4253	Yes	**
MFCE-LD vs. MFCE-HD	-0.2000	-1.175 to 0.7747	No	ns
MFCE-LD vs. INTRASITE	-1.200	-2.175 to -0.2253	Yes	**
MFCE-MD vs. MFCE-HD	1.200	0.2253 to 2.175	Yes	**
MFCE-MD vs. INTRASITE	0.2000	-0.7747 to 1.175	No	ns
MFCE-HD vs. INTRASITE	-1.000	-1.975 to -0.02526	Yes	*
Degeneration (E)				
Control vs. MFCE-LD	1.800	0.8253 to 2.775	Yes	****
Control vs. MFCE-MD	1.800	0.8253 to 2.775	Yes	****
Control vs. MFCE-HD	1.600	0.6253 to 2.575	Yes	***
Control vs. INTRASITE	0.0	-0.9747 to 0.9747	No	ns
MFCE-LD vs. MFCE-MD	0.0	-0.9747 to 0.9747	No	ns
MFCE-LD vs. MFCE-HD	-0.2000	-1.175 to 0.7747	No	ns
MFCE-LD vs. INTRASITE	-1.800	-2.775 to -0.8253	Yes	****
MFCE-MD vs. MFCE-HD	-0.2000	-1.175 to 0.7747	No	ns
MFCE-MD vs. INTRASITE	-1.800	-2.775 to -0.8253	Yes	****
MFCE-HD vs. INTRASITE	-1.600	-2.575 to -0.6253	Yes	***
Granulation Tissue (D)				
Control vs. MFCE-LD	1.400	0.4253 to 2.375	Yes	**
Control vs. MFCE-MD	0.9000	-0.07474 to 1.875	No	ns
Control vs. MFCE-HD	1.200	0.2253 to 2.175	Yes	**
Control vs. INTRASITE	0.9000	-0.07474 to 1.875	No	ns
MFCE-LD vs. MFCE-MD	-0.5000	-1.475 to 0.4747	No	ns
MFCE-LD vs. MFCE-HD	-0.2000	-1.175 to 0.7747	No	ns
MFCE-LD vs. INTRASITE	-0.5000	-1.475 to 0.4747	No	ns
MFCE-MD vs. MFCE-HD	0.3000	-0.6747 to 1.275	No	ns
MFCE-MD vs. INTRASITE	0.0	-0.9747 to 0.9747	No	ns
MFCE-HD vs. INTRASITE	-0.3000	-1.275 to 0.6747	No	ns
Inflammatory Cell (D)				
Control vs. MFCE-LD	1.000	0.02526 to 1.975	Yes	*
Control vs. MFCE-MD	1.300	0.3253 to 2.275	Yes	**
Control vs. MFCE-HD	1.500	0.5253 to 2.475	Yes	***
Control vs. INTRASITE	0.4000	-0.5747 to 1.375	No	ns
MFCE-LD vs. MFCE-MD	0.3000	-0.6747 to 1.275	No	ns
MFCE-LD vs. MFCE-HD	0.5000	-0.4747 to 1.475	No	ns
MFCE-LD vs. INTRASITE	-0.6000	-1.575 to 0.3747	No	ns
MFCE-MD vs. MFCE-HD	0.2000	-0.7747 to	No	ns

		1.175		
		-1.875 to		
MFCE-MD vs. INTRASITE	-0.9000	0.07474	No	ns
		-2.075 to -		
MFCE-HD vs. INTRASITE	-1.100	0.1253	Yes	*

MISSON'S TRICHOM STAIN

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Intensity of coloration				
		-0.8618 to		
Control vs. MFCE-LD	0.1000	1.062	No	ns
		-1.662 to		
Control vs. MFCE-MD	-0.7000	0.2618	No	ns
		-2.062 to -		
Control vs. MFCE-HD	-1.100	0.1382	Yes	*
		-0.5618 to		
Control vs. Intrasite	0.4000	1.362	No	ns
		-1.762 to		
MFCE-LD vs. MFCE-MD	-0.8000	0.1618	No	ns
		-2.162 to -		
MFCE-LD vs. MFCE-HD	-1.200	0.2382	Yes	**
		-0.6618 to		
MFCE-LD vs. Intrasite	0.3000	1.262	No	ns
		-1.362 to		
MFCE-MD vs. MFCE-HD	-0.4000	0.5618	No	ns
MFCE-MD vs. Intrasite	1.100	0.1382 to 2.062	Yes	*
MFCE-HD vs. Intrasite	1.500	0.5382 to 2.462	Yes	***
Fibroblast distribution				
		-2.162 to -		
Control vs. MFCE-LD	-1.200	0.2382	Yes	**
		-2.762 to -		
Control vs. MFCE-MD	-1.800	0.8382	Yes	****
		-2.962 to -		
Control vs. MFCE-HD	-2.000	1.038	Yes	****
		-2.262 to -		
Control vs. Intrasite	-1.300	0.3382	Yes	**
		-1.562 to		
MFCE-LD vs. MFCE-MD	-0.6000	0.3618	No	ns
		-1.762 to		
MFCE-LD vs. MFCE-HD	-0.8000	0.1618	No	ns
		-1.062 to		
MFCE-LD vs. Intrasite	-0.1000	0.8618	No	ns
		-1.162 to		
MFCE-MD vs. MFCE-HD	-0.2000	0.7618	No	ns
		-0.4618 to		
MFCE-MD vs. Intrasite	0.5000	1.462	No	ns
		-0.2618 to		
MFCE-HD vs. Intrasite	0.7000	1.662	No	ns
Collagen maturity				
		-1.162 to		
Control vs. MFCE-LD	-0.2000	0.7618	No	ns
		-1.662 to		
Control vs. MFCE-MD	-0.7000	0.2618	No	ns
		-2.962 to -		
Control vs. MFCE-HD	-2.000	1.038	Yes	****
		-2.162 to -		
Control vs. Intrasite	-1.200	0.2382	Yes	**
		-1.462 to		
MFCE-LD vs. MFCE-MD	-0.5000	0.4618	No	ns
MFCE-LD vs. MFCE-HD	-1.800	-2.762 to -	Yes	****

		0.8382			
MFCE-LD vs. Intrasite	-1.000	-1.962 to -0.03817		Yes	*
MFCE-MD vs. MFCE-HD	-1.300	-2.262 to -0.3382		Yes	**
MFCE-MD vs. Intrasite	-0.5000	-1.462 to 0.4618		No	ns
MFCE-HD vs. Intrasite	0.8000	-0.1618 to 1.762		No	ns
Angiogenesis rate					
Control vs. MFCE-LD	-0.3000	-1.262 to 0.6618		No	ns
Control vs. MFCE-MD	-1.500	-2.462 to -0.5382		Yes	***
Control vs. MFCE-HD	-1.100	-2.062 to -0.1382		Yes	*
Control vs. Intrasite	-0.7000	-1.662 to 0.2618		No	ns
MFCE-LD vs. MFCE-MD	-1.200	-2.162 to -0.2382		Yes	**
MFCE-LD vs. MFCE-HD	-0.8000	-1.762 to 0.1618		No	ns
MFCE-LD vs. Intrasite	-0.4000	-1.362 to 0.5618		No	ns
MFCE-MD vs. MFCE-HD	0.4000	-0.5618 to 1.362		No	ns
MFCE-MD vs. Intrasite	0.8000	-0.1618 to 1.762		No	ns
MFCE-HD vs. Intrasite	0.4000	-0.5618 to 1.362		No	ns
Collagen distribution					
Control vs. MFCE-LD	-0.1000	-1.062 to 0.8618		No	ns
Control vs. MFCE-MD	-1.600	-2.562 to -0.6382		Yes	***
Control vs. MFCE-HD	-1.900	-2.862 to -0.9382		Yes	****
Control vs. Intrasite	-0.7000	-1.662 to 0.2618		No	ns
MFCE-LD vs. MFCE-MD	-1.500	-2.462 to -0.5382		Yes	***
MFCE-LD vs. MFCE-HD	-1.800	-2.762 to -0.8382		Yes	****
MFCE-LD vs. Intrasite	-0.6000	-1.562 to 0.3618		No	ns
MFCE-MD vs. MFCE-HD	-0.3000	-1.262 to 0.6618		No	ns
MFCE-MD vs. Intrasite	0.9000	-0.06183 to 1.862		No	ns
MFCE-HD vs. Intrasite	1.200	0.2382 to 2.162		Yes	**

APPENDIX E: LIST OF PUBLICATION AND PAPERS

Shaymaa Fadhel Abbas, Yusuf Abba, Abdullah Rasedee and Noorlidah Abdullah. (2016). Prophylactic effects of *Clausena excavata* Burum. F leaf extract in ethanol-induced gastric ulcer. Drug Design, Development and Therapy. 2016: 10:1973-86

Shaymaa Fadhel Abbas, Yusuf Abba, Abdullah Rasedee and Noorlidah Abdullah. (2015). Effect of *Clausena excavata* Burm. f. (Rutaceae) leaf extract on wound healing and antioxidant activity in rats. Drug Design, Development and Therapy. 2015:9 3507–3518.

Shaymaa Fadhel Abbas, Yusuf Abba, Abdullah Rasedee and Noorlidah Abdullah. (2014). Evaluation of antioxidant activity and acute toxicity of *Clausena excavata* leaves extract. Evid Based Complement Alternat Med. 2014; 2014:975450.

In Vitro Evidence of Anti-Inflammatory Properties of bioactive methanolic fraction of *Clausena excavata*. INTERNATIONAL CONFERENCE OF PHARMACY AND HEALTH SCIENCES 2016.

Anti-inflammatory evidence of *Clausena excavata* in excision wound-induced rats. MALAYSIAN SOCIETY OF ANIMAL PRODUCTION 2016.

The Ethics Committee for Animal Experimentation, Faculty of science University of Malaya, Malaysia (Ethic No.ISB/22/07/2013/SFA (R)).

UNIVERSITI MALAYA

UM.C/TNC2/625/16/9/1

22 July 2013

Shaymaa Fadhel Abbas
Institute of Biological Sciences
Faculty of Science
University of Malaya

Mr/ Madam,

Title Project: **ANTIMICROBIAL, ANTI-OXIDANT, WOUND HEALING POTENTIAL AND ANTIULCER ACTIVITY OF SELECTED MEDICINAL PLANT AGAINTS NECROTIZING AGENTS-INDUCED GASTRIC MUCOSAL INJURY IN EXPERIMENTAL RATS**

This is to kindly inform you that the Institutional Animal Care and Use Committee, University of Malaya (UM IACUC) has approved your Animal Research Protocol Application (ARPA) for duration of two (2) years effective from **22 July 2013** until **22 July 2015**.

Your Ethics Reference no. : **ISB/22/07/2013/SFA (R)**

Thank you.

Yours sincerely,

Durriyyah SHHA

Assoc. Prof. Dr. Durriyyah Sharifah Hasan Adli
Chairperson
Institutional Animal Care and Use Committee (IACUC)
University of Malaya

ASSOC. PROF. DR. DURRIYYAH SHARIFAH HASAN ADLI
Chairperson
Institutional Animal Care and Use Committee, University of Malaya (UM IACUC)

JAWATANKUASA INSTITUSI PENJAGAAN DAN PENGGUNAAN HAIWAN UNIVERSITI MALAYA (UM IACUC)
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Drug Design, Development and Therapy

Dovepress

ORIGINAL RESEARCH

Prophylactic effects of *Clausena excavata* Burum. f. leaf extract in ethanol-induced gastric ulcers

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Yusuf Abba[3]
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Abstract: *Clausena excavata* is a natural herb with both antioxidant and anti-inflammatory properties. It has been used for decades in folkloric practice for the amelioration of various ailments. In this study, the gastroprotective activity of methanolic extract of *C. excavata* leaves (MECE) was determined in the Sprague Dawley rat ethanol-induced gastric ulcer model. Rats were pretreated with a single dose of vehicle (5% Tween 20), 20 mg/mL omeprazole, 400 and 200 mg/mL of MECE dissolved in 5% Tween 20. Ulcer was induced with 5 mL/kg of ethanol and stomach tissue was obtained after 1 hour. Histological examination was done on hematoxylin and eosin, periodic acid-Schiff, and immunochemically stained gastric mucosal tissues. Prostaglandin E2, superoxide dismutase, catalase, glutathione peroxidase, and lipid peroxidation levels of the gastric tissue homogenates were also determined. Significantly ($P<0.05$) smaller ulcer areas, less intense edema, and fewer leukocytes' infiltration were observed in MECE- and omeprazole-treated than in untreated gastric mucosa with ulcer. The gastric pH, mucus production, superoxide dismutase, catalase, and glutathione peroxidase contents increased, while the lipid peroxidation content decreased as a result of MECE treatment. Bcl-2-associated X protein was underexpressed, while heat shock protein 70 and transforming growth factor-beta protein were overexpressed in the ulcerated gastric mucosa tissues treated with omeprazole and MECE. Similarly, there was a reduction in the levels of tumor necrotic factor-alpha and interleukin-6, while the level of interleukin-10 was increased. This study showed that the gastroprotective effect of MECE is achieved through inhibition of gastric juice secretion and ulcer lesion development, stimulation of mucus secretion, elevation of gastric pH, reduction of reactive oxygen species production, inhibition of apoptosis in the gastric mucosa, and modulation of inflammatory cytokines.

Keywords: Rutaceae, antiulcer, antioxidant enzymes, histopathology, immunohistochemical proteins, cytokines

Introduction

Peptic ulcer disease (PUD), a protracted and multifactorial disease, is characterized by the presence of ulcerated lesions in the gastric or duodenal mucosa. PUD occurs in approximately 20% of the world population.[1] Development and progression of PUD is governed by the acidity of the stomach's gastric juice. Thus, modulation of the stomach acidity is fundamental to the prevention and healing of gastric ulcers.[2] Although several antiulcer medications such as H2 receptor antagonists (ranitidine, famotidine), antacid, and proton•pump inhibitors (omeprazole) are readily available for the treatment of ulcers, these therapeutic agents have side effects that include impaired calcium, iron, magnesium, and vitamin B12 absorption in the intestine. Hence, there is a great need to develop more efficacious drugs for the treatment of the disease.[3]

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Drug Design, Development and Therapy

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ORIGINAL RESEARCH

Effect of *Clausena excavata* Burm. f. (Rutaceae) leaf extract on wound healing and antioxidant activity in rats

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Abstract: *Clausena excavata* is a well-known plant used in folkloric medicine for the treatment of different ailments. This study aimed to determine the in vitro cytoxicity of its leaf solvent extracts as well as the in vivo wound healing and antioxidant activities of the methanolic extracts of *C. excavata* (MECE). HaCaT (keratocyte) and Vero cell lines were used for evaluation of the in vitro cytotoxic effects, while the in vivo wound healing and antioxidant activities were determined in skin wounds inflicted on rats. Twenty adult male Sprague-Dawley rats were divided into five groups of four animals each. Approximately 3.14 cm^2 excisional wound was inflicted on the nape of each rat following anesthesia. The treatment groups received topical application of MECE at 50 mg/mL (MECE-LD [low dose]), 100 mg/mL (MECE-MD [medium dose]), and 200 mg/mL (MECE-HD [high dose]), while the negative control group was treated with gum acacia in normal saline and the positive control group with intrasite gel. Wound contraction was evaluated on days 5, 10, and 15 after wound infliction, and tissue from wound area was collected at day 15 post-wound infliction for antioxidant enzyme evaluation and histopathological analyses. Generally, Vero cells were more resistant to the cytotoxic effects of the solvent extracts as compared with HaCaT cells. Chloroform (CH) and ethyl acetate (EA) extracts of *C. excavata* were toxic to HaCaT cells at 200 and 400 µg/mL, but the same concentrations showed higher ($P<0.05$) viability in Vero cells. There was significantly ($P<0.01$) greater wound contraction at days 10 and 15 post-wound infliction in all the treatment groups than in the control groups. Histopathologically, the MECE-HD-treated wound showed significantly ($P<0.05$) lesser inflammatory cell proliferation, degeneration, and distribution of granulation tissue than other groups. Similarly, the degree of collagen maturation, angiogenesis, and collagen distribution were significantly ($P<0.05$) lower in MECE-HD than in other groups. The MECE-HD, MECE-MD, and intrasite treatment groups showed a significantly ($P<0.05$) higher number of VEGF-positive and TGF-β1-positive cells in the skin wound than the control groups. The activities of superoxide dismutase and catalase were significantly ($P<0.01$) higher in the MECE-HD and intrasite treatment groups than in the other groups. Lipid peroxidase activity of the treated groups was significantly ($P<0.01$) lower than that in the control group. The study showed that MECE is a potent wound healing agent through anti-inflammatory and antioxidant effects that enhanced the rate of wound contraction, re-epithelialization, and collagen deposition. The effect of MECE is suggested to be due to its high polyphenolic compound content.

Keywords: *Clausena excavata*, cytotoxicity, wound healing, antioxidant, histopathology, VEGF, TGF-β1

Introduction

Clausena excavata Burm. f. is one of the potherb species with high antioxidant properties. Its leaves and stem are used in folk medicine for treatment disorders such

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Hindawi

Research Article

Evaluation of Antioxidant Activity and Acute Toxicity of *Clausena excavata* Leaves Extract

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Clausena excavata (Lour.), locally known as "Kemantu hitam," is a common plant in Malaysian folklore medicine. This study evaluated the antioxidant properties of the solvent extracts of *C. excavata* leaves and determined the acute toxicity of methanolic extract *C. excavata* (MECE) leaves in Sprague-Dawley rats. Harvested leaves were dried and subjected to solvent extraction using petroleum ether, chloroform, ethyl acetate and methanol in succession. The antioxidant activity of each extract was determined using the ferric-reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picryl dihydrazyl (DPPH) radical scavenging activity. The total phenolic content (TPC) and total flavonoids content (TFC) were estimated by Folin-Ciocalteu and ethanolic aluminium chloride method, respectively. The chloroform extract was found to be highest in flavonoid content, while the methanolic extract showed the highest TPC and antioxidant activity. There was no mortality in rats treated with MECE leaves even at a high dose of 5000 mg/kg body weight. However, the MECE leaves produced mild to moderate pathological changes in the liver and kidneys, shown by mild degenerative changes and leucocyte infiltration. The extract did not affect the haematological parameters or relative weights of the liver or kidneys. Overall, the MECE leaves have potent antioxidant activity and are presumed safe to be used orally as health-promoting product at low to moderate doses.

1. Introduction

Free radicals are harmful by-product of cellular oxidative phosphorylation and energy production. These radicals damage various intracellular macromolecules to include DNA, protein, and lipids [1]. Antioxidants have the ability to prevent oxidative damage and inhibit inflammatory conditions by nullifying the activities of free radicals [2]. Currently, the search for plant sources of antioxidants is gaining momentum with *Clausena excavata* (Rutaceae family) among the plants targeted. *C. excavata* is a medicinal plant widely distributed in Southeast Asia and is known by unique local names, such as Chamat in Thailand and Jia huang pi in China. In Malaysia the plant is locally known as "Cherek hitam" and "Kemantu hitam." The leaves of the plant are used in folklore medicine for the treatment of several illnesses such as malaria, headache, abdominal pain, dysentery, pulmonary tuberculosis, diarrhoea, cold, wound, snake-bite, and poisoning. Recent studies showed that the plant also possessed immune-modulatory [3], analgesic [4], anti-inflammatory, antivirus, anticancer [5], antioxidant [6], antimycobacterial [7], and antifungal [8] activities. *C. excavata* has been reported to exhibit one of the highest beneficial biological activities among *Clausena* genus [9]. These activities are attributed to its high phenolic compounds such as furanocoumarins and flavonoids. The plant also contains other pharmacologically active compounds like coumarin, carbazole alkaloid, and flavonoid glycosides [7, 10–12].

It is imperative that compounds from plants for human use are screened for potential toxicity, particularly for