EVALUATION OF GASTRO PROTECTIVE EFFECT AND WOUND HEALING POTENTIAL OF CIBOTIUM BAROMETZ AND VITEX PUBECSENS IN AN ANIMAL MODEL

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ABSTRACT

*Cibotium barometz* and *Vitex pubescens* are Malaysian medicinal plants used traditionally as remedies for gastrointestinal ailments and wounds. The present study aimed to assess the gastro protective effects and wound healing potential of both leaves of plants. In vitro, their ethanol extracts showed high antioxidant capacities with no cytotoxic effects on WRL-68 cell line. The active compounds identified were 24-Methylenecycloartanol in *C. barometz* and Vitexin with Luteoloside in *V. pubescens*. Down regulation of *Bax* and up regulation of *Col1a2* genes were observed in Hs27 fibroblast cells treated with *C. barometz*. *In vivo*, neither hepatotoxicity nor nephrotoxicity was detected in the acute toxicity test of treated Sprague Dawley (SD) rats with each plant. In a dose-dependent manner, the rats pre-treated with each extract prior to being ethanol induced with gastric ulcers showed significant decreasing of ulcer area, moderate to mild disruption of the surface epithelium with reduction in submucosal oedema and leucocyte infiltration in the gastric tissue comparing to ulcer control group. Moreover, Periodic acid Schiff (PAS) stain demonstrated an increasing in glycoprotein content of gastric mucus barrier, over expression of Heat Shock Protein 70 (HSP70) and down-expression of Bcl-2-associated X protein (Bax) in rats pre-fed with each plant. Gastric tissue homogenates revealed significant increase of superoxide dismutase activity (SOD) and decrease of malondialdehyde (MDA) levels. In the wound healing studies, the normal wounds of each rat that dressed with *C. barometz* and *V. pubescens* leaves demonstrated a reducing of the wound closure area and acceleration of the wound healing potential in a dose independent manner and time of dressing. Moreover, the granulation tissues of wounds stained by hematoxylin and eosin (H&E) displayed the increases of fibroblast proliferations, vascularization, and collagen depositions with fewer inflammatory cells. In addition, Masson’s trichrome (MT) staining of each dressed rat demonstrated high collagen accumulations and generations
of new capillary vessels of the endothelial cells in the dressed rats when compared to carboxymethyl cellulose (CMC 2%) of vehicle group. The protein expressions of HSP70 was up regulated but Bax was down regulated were down regulated, elevated of SOD endogenous enzyme activities and dropped of MDA levels of normal skin wounds were exhibited in normal rats compared to the vehicle group.

However, the results of diabetic wound had more time to be healed than normal wounds in the rats of experiments as a result of delaying in the chronic diseases. The diabetic wounds in rats, which dressed with *C. barometz* and *V. pubescens* leaves showed a lower of wound closure area and acceleration of the wound closure percentage of wounds in a dose independent manner and time of healing. Moreover, the granulation tissues of wounds using H&E staining exhibited the increases of fibroblast proliferations, vascularization, and collagen depositions with fewer inflammatory cells. Additionally, MT staining of treated rats demonstrated high collagen accumulations and generations of new capillary vessels of the endothelial cells in the dressed rats when compared to carboxymethyl cellulose (CMC 2%) of vehicle group. Conversely, the protein expressions of HSP70 was up regulated and Bax was down regulated of the skin tissue, elevated of SOD endogenous enzyme activities and dropped of MDA levels of diabetic rats were exhibited compared to the vehicle group. Therefore, the accelerated healing of wounds and ulcers in this study may be as a result of each plant in this study, which had antioxidant and anti-inflammatory properties particularly, *C. barometz*. 
ABSTRAK

*Cibotium barometz* dan *Vitex pubescens* adalah tumbuhan perubatan Malaysia yang digunakan secara tradisional sebagai ubat untuk penyakit gastrousus dan luka. Kajian ini bertujuan untuk menilai kesan perlindungan gastro dan luka potensi kedua-dua daun tumbuh-tumbuhan penyembuhan. *In vitro*, ekstrak etanol mereka menunjukkan kapasiti antioksidan yang tinggi dengan tiada kesan sitotoksik pada WRL-68 garis sel. Sebatian aktif yang dikenal pasti adalah 24 Methyleneoartanol dalam *C. barometz* dan Vitexin dengan Luteoloside dalam *V. pubescens*. Down peraturan Bax dan sehingga peraturan gen Col1a2 diperhatikan dalam sel-sel fibroblast Hs27 dirawat dengan *C. barometz*. Dalam vivo, tidak hepatoksisiti tidak nephrotoxicity dikesan dalam ujian ketoksikan akut dirawat Sprague Dawley (SD) tikus dengan setiap pokok. Dengan cara yang bergantung kepada dos, tikus pra-dirawat dengan ekstrak sebelum ianya etanol yang disebabkan ulser gastrik menunjukkan ketara mengurangkan kawasan ulser, sederhana kepada gangguan ringan epitelium permukaan kerja dengan pengurangan submucosal edema dan Leucocyte penyusupan dalam tisu gastrik berbanding dengan ulser kumpulan kawalan. Selain itu, asid berkala Schiff (PAS) noda menunjukkan peningkatan dalam kandungan Glikoprotein halangan mukus perut, lebih ungkapan Heat Shock Protein 70 (HSP70) dan turun-ungkapan protein X Bcl-2-berkaitan (Bax) pada tikus pra-makan dengan setiap pokok. homogenates tisu gastrik mendedahkan peningkatan ketara aktiviti superoxide dismutase (SOD) dan penurunan tahap malondialdehid (MDA). Dalam kajian penyembuhan luka, luka-luka yang biasa setiap ekor tikus yang berpakaian dengan *C. barometz* dan *V. pubescens* daun menunjukkan yang mengurangkan daripada kawasan penutupan luka dan mempercepatkan luka potensi penyembuhan dengan cara bebas dos dan masa persalinan. Selain itu, tisu granulation luka yang dicemarkan oleh hematoxylin dan eosin (H & E) yang dipaparkan
meningkat daripada proliferations fibroblast, vascularization dan deposisi kolagen
dengan sel-sel radang yang lebih sedikit. Di samping itu, trichrome Masson (MT)
mengotorkan setiap ekor tikus yang berpakaian menunjukkan pengumpulan kolagen
yang tinggi dan generasi kapal kapilari baru sel-sel endothelial dalam tikus berpakaian
berbanding carboxymethyl selulosa (CMC 2%) daripada kumpulan kenderaan.
Ungkapan protein HSP70 naik dikawal selia tetapi Bax telah turun dikawal selia telah
turun dikawal selia, tinggi aktiviti enzim SOD dalaman dan jatuh tahap MDA luka kulit
normal telah dipamerkan pada tikus normal berbanding dengan kumpulan kenderaan.

Walau bagaimanapun, keputusan luka kencing manis mempunyai lebih banyak masa
untuk sembuh daripada luka normal dalam tikus eksperimen akibat melambatkan dalam
penyakit kronik. Luka diabetes pada tikus, yang berpakaian dengan \textit{C. barometz} dan \textit{V. pubescens}
daun menunjukkan yang lebih rendah luka kawasan penyembuhan dan
pecutan peratusan penyembuhan luka dengan cara bebas dos dan masa penyembuhan.
Selain itu, tisu granulation luka menggunakan H & E pewarnaan dipamerkan kenaikan
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pengumpulan kolagen yang tinggi dan generasi kapal kapilari baru sel-sel endothelial
dalam tikus berpakaian berbanding carboxymethyl selulosa (CMC 2%) daripada
kumpulan kenderaan. Sebaliknya, ungkapan protein HSP70 naik dikawal selia dan Bax
telah turun dikawal selia tisu kulit, tinggi daripada aktiviti-aktiviti enzim SOD dalaman
dan jatuh tahap MDA tikus kencing manis telah dipamerkan berbanding dengan
campuran kenderaan. Oleh itu, penyembuhan dipercepatkan luka dan ulser dalam kajian
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AlCl$_3$</td>
<td>Aluminum chloride</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>Bcl$_2$</td>
<td>B-cell lymphoma 2</td>
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<td>BMM</td>
<td>Bone marrow-derived macrophages</td>
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<td>CAMs</td>
<td>Cellular adhesion molecules</td>
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<td>Catalase</td>
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<td>ΔΔC$_T$</td>
<td>Delta delta cycle threshold</td>
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<td>DFUS</td>
<td>Diabetic foot ulcers</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DPPH</td>
<td>1,1-Diphenyl-2-picrylhydrazyl radical</td>
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<tr>
<td>DPX</td>
<td>Digital picture exchange</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix (ECM)</td>
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<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
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<td>EGF</td>
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<td>Serum enzyme-linked immunosorbent assay</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>HRE</td>
<td>Hypoxia response element</td>
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Hs27 Human skin fibroblast cell line
HSF-1 Heat shock factor-1
HSP70 Heat shock protein 70
IC$_{50}$ Inhibitory concentration
IBS Institute of Biological Sciences
IDDM insulin-dependent diabetes mellitus
IGF-1 Insulin like growth factor 1
IL-8 Interleukin 8
Kb kilobite
KDa Kilodalton
KGF Keratinocyte growth factor
L Liter
LC-MS Liquid chromatography mass spectrometry
m/z Mass / charge number of ions
MALT Mucosa-associated lymphoid tissue
MCP1 Macrophage chemoattractant protein 1
MDA Malondialdehyde
MIP2 Macrophage inflammatory protein 2
ML Milliliter
MMPs Matrix-metalloproteases
MT Masson’s trichrome
MTT 3-[4, 5- Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
N$_2$O$_3$ Dinitrogen trioxide
NADH Nicotinamide adenine dinucleotide, reduced disodium salt hydrate
NBT Nitro blue tetrazolium
Ng
Nano gram

NIDDM
Non-insulin-dependent diabetes mellitus

nm
Nanometre

NO•
Nitric oxide

NO₃
Nitrate

NSAIDS
Non-steroidal anti-inflammatory drugs

O₂•
Superoxide anion

OECD
Economic Cooperation and Development

PDGF
Platelet derived growth factor

PAS
Periodic acid–Schiff

PBS
Phosphate-buffered saline

Pb
Lead

%
Percentage

PGs
Prostaglandins

PMS
Phenazine methosulfate

PPIs
Proton pump inhibitors

RANTES
Regulated on activation normal T cell expressed and secreted

RNA
Ribonucleic acid

RNS
Reactive nitrogen species

ROS
Reactive oxygen species

rpm
Rotation per minute

RPMI
Roswell park memorial institute

RT
Reverse transcription

RT-PCR
Real time-polymerase chain reaction

S
Small subunits

SA
Sulfanilamide
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<tr>
<td>SD</td>
<td>Sprague Dawley</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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CHAPTER 1: INTRODUCTION

1.1 Background

1.1.1 Gastric ulcer

Gastric ulcers are benign lesions of the gastric mucosa that affect many people around the world (Diniz et al., 2013). It is caused by the imbalance between known aggressive factors and the mucosal defence mechanisms in the mucosal epithelium (Shaker, 2010). There are a lot of factors that contribute to enhance the incidence of gastrointestinal disorders such as stress, smoking, alcohol consumption, nutritional deficiencies and ingestion of non-steroidal anti-inflammatory drugs (NSAIDS), hereditary predisposition and infection by *Helicobacter pylori* (Ji et al., 2012). Many people take various drugs to support the mucosal defense mechanisms in the stomach and treat the gastric ulcer such as proton pump inhibitors (omeprazole), antacids, antihistaminic agents or NSAIDS such as aspirin, ibuprofen, diclofenac, etc. However, they have many adverse side effects (Silva et al., 2013). Sometimes anti-acid drugs are unsuccessful and impair the absorption of acid gastric medium requirements of calcium, iron, magnesium and vitamin B12 for bioavailability. Medicinal plants can assist in ulcer and wound healing and in preventing recurrence that are used as alternative treatments (Abdelwahab et al., 2013). Nowadays, the alternative medicine and the medicinal plants used to prevent the recurrence of gastric ulcers and treat many disorders without side effects. The herbal medicine can assist in ulcer and wound healing through increasing the scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and decreasing the free radical cytotoxicity of gastrointestinal membrane (Tuluce et al., 2011).
1.1.2 Wound healing

Wound healing is a dynamic, intricate, and auto process of injured site skin encompassing biochemical, cellular, molecular, and physiological events (Delavary et al., 2011). The injured skin and wounds are very common among humans that caused through mechanical, chemical, electrical, thermal, or nuclear sources. While the skin is the first line protector of body against pathogens, the expeditiously repair of wounds is principal to survival and lessen the complications (Yanik et al., 2015). This auto process including four main phases: hemostasis and coagulation, inflammation, proliferation and maturation or tissue remodelling as shown in Figure 1.1. It is regulated by wound mechanisms such as differentiated cells, stem cells, cytokine networks, extracellular matrix, and mechanical forces (Al-Bayaty & Abdulla, 2012). The effectiveness mechanism of the wound healing is reported to be highly dependent on the proper sequential occurrence of the four phases within a specified time frame (Guo & DiPietro, 2010). However, the impairment and delaying of the healing process have been appeared in many chronic diseases, such as diabetes mellitus (Hou et al., 2013). Diabetes mellitus is one of the greatest prevalence diseases that are defined as a chronic state of hyperglycemia with alterations in the metabolism of proteins, fats, and carbohydrates. Uncontrolled diabetes has been associated with a longer inflammation stage, defects in angiogenesis, and reduction of fibroblast proliferation. Diabetic foot ulcers occur in 15% of all patients with diabetes and precede 84% of all diabetes-related lower leg amputations (Heublein et al., 2015).

For wound repair shortly and management of wounds with less undesired consequences, there are different drugs, vehicles and treatment modalities have been used locally or systemically. They include antiseptic, antibiotic creams, gels, occlusive layers, bandages, poultices and mechanical devices that reduce evaporation of water and others (Kant et al., 2014). However, the existing drugs do not restore normal
glucose homeostasis and they have many side effects. Hence natural products and medicinal plants are found safer and useful in treating wounds and ulcers (Shukla et al., 2010).

**Figure 1.1**: Overview of the wound healing process (Borena et al., 2015).

### 1.1.3 Medicinal plants

The medicinal plants have been used as a preventative and alternative medicine for various ailments since ancient years. They have several parts and many medicinal properties. Good therapeutic performance and low toxicity of plants enhance their potential medicinal value and production of new drugs (Hajiaghaalipour et al., 2013). Recently, major pharmaceutical companies in the world have conducted extensive research on therapeutic plants that are assembled from the rain forests and other places for their potential medicinal value (Hossain & Nagooru, 2011). Many pharmaceutical plants such as *Bauhinia thonningii*, *Polygonum minus*, *Andrographis paniculata*, *Curcuma xanthorrhiza* are used for prevention and treatment many ailments such as gastrointestinal disorders, wounds, cystitis, diabetes, headaches, hypertension,
insomnia, liver problems (Al Bayaty et al., 2010; De Sousa et al., 2010; Abdelwahab et al., 2013).

1.1.3.1 Cibotium barometz

*Cibotium barometz* (L.) J. Sim. (Family Dicksoniaceae) is known as Golden Hair Dog Fern. It is a tropical and subtropical plant in the Malay Peninsula and parts of China is used commonly in traditional medicine (Bobach et al., 2014). In natural remedies, the yellow hairs and the rhizomes of plant are used in poultices and ointments on wounds to stop bleeding due to the anti inflammation effects. Moreover, the dried rhizomes that are taken orally are used as an anti-hepatitis virus agent and demonstrated hepatoprotective activities on tacrine-induced cytotoxicity in human liver-derived Hep G2 cells (Wu & Yang, 2009). There are many phenolic compounds in *Cibotium* that showed potent antioxidants and strong chelating power (Wu & Yang, 2009). It is reported by Syafni et al., (2012) presence of 3,4-dihydroxybenzoic acid and 3,4-dihydroxybenzaldehyde present in this fern exhibited antimicrobial properties. This medicinal plant that applied in natural remedies is used in traditional healing and stop bleeding. In addition, it is used for the treatment of rheumatism, polyuria, rheumatic and menstruation problems, herniated disc and hyperosteogeny and leucorrhoea (Cuong et al., 2009; Bobach et al., 2014). *C. barometz* inhibits osteoclast creation with no effect on Bone Marrow-Derived Macrophages (BMM) cell viability and has a kidney-tonifying activity that is one of the most frequently used herbs in formulas that prescribed for the treatment of osteoporosis. Its extract has potential for further development as a natural alternative in the management of postmenopausal osteoporosis (Zhao et al., 2011).
1.1.3.2 *Vitex pubescens*

*Vitex pubescens* Vahl. (Verbenaceae), is known as halban or laban in the Peninsula of Malaysia and has another name that is *Vitex pinnata* (Ng, F. (1989); Ong, H., & Nordiana, M. (1999). The genus *Vitex* includes about 270 known species of shrubs and trees in tropical and sub-tropical regions. The people have used some *Vitex* species like, *Vitex pubescens*, *Vitex trifolia*, *Vitex paniculata* and *Vitex parviflora* for traditional medicine. Many studies conducted to date have revealed that *Vitex* plants comprise many bioactive compounds biologically with, anti-inflammatory, antioxidant, antimicrobial, hepatoprotective, analgesic antihistamine, anti-asthmatic, and anti-implantation effects (Meena *et al*., 2010). *V. pubescens* has anti-dysentery, anti-inflammatory, analgesic, antipyretic, anti-fungal and antitumor activities (Meena *et al*., 2010; Oramahi & Yoshimura, 2013).

It is used in the treatment of gastrointestinal diseases and the leaf paste of is applied on wounds (Batubara *et al*., 2009). This genus has been used for traditional medicines such as menstruation, gynaecology and for bug repellent, fungi and bacteria. *V. pubescens* leaves and its stem bark have been used for lumbago, cut, indigestion, fever, scorpion sting, increasing appetite, dysentery, anti-inflammation, cancer and rhinitis, and also for stamina (Lenny *et al*., 2015). The young leaf of this plant is consumed as raw to counter hypertension and fever. In addition, the root-tea has been used for backache, bodyache and fatigue. It has many biological compounds such as pinnatasterone, andrographolide, 20-hydroxyecdysone and turkesterone, luteolin, iso-orientin, and vitexin, methyl p-hydroxybenzoate. These compounds showed a low biological activity with housefly larvae in the pupariation test, antifungal in food and used for many cosmetic products (Meena *et al*., 2010; Lenny *et al*., 2015).
1.2 Problem statement

The widespread use of synthetic drugs and their management remains the single most serious complication of any drug therapy. As the recommended anti-ulcer drugs such as omeprazole and others show several adverse effects and are more expensive than non-synthetic drugs (Fahmy et al., 2015). Furthermore, synthetic drugs that are used in wound healing either cutaneous wounds or chronic wounds such as in diabetic disease confer a frequent recurrence and an inadequate healing (Romero-Cerecero et al., 2014). Therefore, health care resources are unsuccessfully used worldwide and alarmingly increasing in the expenditure of the total health care. In this regard, substantial efforts and many researches in the scientific community have been made and focused on finding new affordable and safe formulations (Gainza et al., 2015). Creation of effective and affordable medicines from herbal and medicinal plants is an essential goal in gastrointestinal pharmacology and wound treatments and an alternative approach to control the disease (AlRashdi et al., 2012).

1.3 Objectives

1.3.1 General objectives

This current study was designed for evaluation the gastro-protective effect and the wound healing potential of ethanol extracts of *C. barometz* and *V. pubescens* leaves in SD rats.

1.3.2 Specific objectives

1) To assess the anti-oxidant properties of *C. barometz* and *V. pubescens* leaves ethanol extracts.

2) To evaluate the cytotoxicity effects of *C. barometz* and *V. pubescens* leaves ethanol extracts on cell lines, *in vitro*. 
3) To fractionate each plant for bioactivity tests and identify the active chemical constituents in ethanol extracts of each plant.

4) To evaluate the changes of the higher effective plant in gene expression of apoptosis markers (*Bax* and *Col1a2*) in fibroblast (skin cell), *in vitro*.

5) To assess the acute toxicity of each plant in SD rats.

6) To evaluate the anti-ulcer activity of each plant extracts on ethanol-induced gastric ulcer in experimental rats grossly, histologically (H&E and PAS staining), and immunohistochemistry.

7) To evaluate the wound healing potential of each plant on normal and diabetic excision wounds in experimental rats grossly, histologically (H&E and Masson’s trichrome staining) and immunohistochemistry.

8) To assess the superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities and malondialdehyde (MDA) levels of gastric and wound tissues homogenates in the experimental animals.

### 1.4 Study scope

*C. barometz* and *V. pubescens* leaves are screened to reveal their medicinal properties and evaluated to quantify their effective biological actions. The major scope of this study concern the evaluation of the antioxidant activities, cytotoxicity effects, gastro protective activities and wound healing potential in rats for both plants. This thesis is divided into five chapters to confirm an appropriate and easy illustration of the concept of the research:

Chapter 1 illustrates the overview of the gastric ulcers, wound healing issues and the medicinal plants of study.

Chapter 2 presents the literature review of the main subjects in this study.
Chapter 3 elucidates the materials and the methodology of the research.

Chapter 4 demonstrates the findings of analysis and results in this project.

Lastly, Chapter 5 displays the discussion and the conclusions of this study.
CHAPTER 2: LITERATURE REVIEW

2.1 Gastrointestinal tract disorders

The gastrointestinal tract (GIT) is one of the most essential and complex organ system in the humans for digesting, absorbing nutrients and excreting the undigested food. GIT comprises the esophagus, stomach (has fundus, corpus and antrum-pylorus), small intestine and colon segments with two main divisions: the upper and lower tracts including three main regions: foregut, midgut and hindgut as shown in Figure 2.1 (San Roman & Shivdasani, 2011; Bahmani et al., 2014). It has a defense function against numerous pathogens related to extreme different disorders such as parasitic and infectious diseases, diarrhea, gastroenteritis, constipation, bloating and peptic ulcers (Jesus et al., 2013).

Figure 2.1: Gastrointestinal tract regions (Adapted from San Roman & Shivdasani, 2011).
Peptic ulcers affect millions of people worldwide. It is one of the most common gastro-intestinal disorders. According to the report of World Health Organization, one out of ten Americans has peptic ulcers during the lifetime. (Hilmarsdottir et al., 2012). A peptic ulcer is an erosion in the duodenal or gastric mucosal barrier that occurs with increasing of gastric acid production (Oppong et al., 2015). Nowadays, the ulcer in the stomach is becoming one of the medical–social problems of global economic importance due to its higher morbidity and mortality (Ji et al., 2012).

2.1.1 Gastric ulcer definition

Gastric ulcer is one of the gastrointestinal tract disorders that have become a common public health worldwide with increasing prevalence. Furthermore, rapid improvement and civilizational constraints in the world have created higher incidence of gastric ulcers. Therefore, severe complications of gastric ulcers such as haemorrhages, perforations, gastrointestinal obstructions, and gastric cancers increase the morbidity and mortality. It is estimated that 14.5 million people in the world are affected by gastric ulcer with a mortality rate of 4.08 million (Chen et al., 2015; Yu et al., 2015).

Gastric ulcers are defects in the gastric barrier that extend through the mucosal epithelium tissue into deeper layers of stomach (mucosa, submucosa, muscle layers, serosa) as illustrated in Figure 2.2. They are pluricausal diseases and developed from imbalances between several factors (Choi et al., 2013). The gastric ulcer aetiology remains indistinct and in spite of extensive research, it is likely that the cause of ulcer varies among individuals as a result of multi processes for controlling the disease (Silva & de Sousa, 2011; Laloo et al., 2013).
2.1.2 Gastric ulcer pathology and etiology

Gastric ulcer has multietiopathological factors that are caused by decreasing the protection mechanism efficacy in the stomach. Therefore, a complex epithelium has layered the normal stomach and creates particular barriers and also produces mucus, bicarbonate, generated prostaglandins, and heat shock proteins for stomach protections (Tuluçe et al., 2011). The barriers are between the internal and external of the body in the stomach to conserve the structural and functional integrity of the mucosa and avoid the destructive pathogens. In the stomach ulcer, the main defence function of gastric mucosal barriers has been penetrated by different factors to provoke the disease. Silva & de Sousa (2011) mentioned that the gastric mucosal barriers include three main barriers:

1. Pre epithelial mucus-bicarbonate barrier (the first line of mucosal defence):

It contains the viscoelastic mucus gel that acts as an antioxidant agent and decreases the gastric mucosa destruction. Intracellular mucus has approximately 5% mucin glycoproteins and 95% water to cover the entire gastric mucosa. The produced mucus acts as a lubricating constituent that reduces the physical injury to the epithelium via swallowed materials; trapped the bacteria. It also assists the maintenance of the acidity in the stomach to be a neutral pH at its epithelial surface. (Sperandio et al., 2015). In
addition, the first line barrier has the bicarbonate that neutralizes acid distribution at the surface interface of mucus-mucosal and surfactant phospholipids that have robust hydrophobic properties and cover the luminal surface of stomach. Moreover, the released pepsinogen (polypeptide proenzyme) is activated to pepsin by the acid milieu in the stomach for the hydrolysis of dietary proteins, assists in destroying the ingested bacteria and aids the mucosal defence function. Therefore, the gel structure and the thickness of mucus play an important role in the gastric defence efficacy of mucus barrier (Osaki et al., 2010; Boltin & Niv, 2014).

2. Epithelial barrier (the second line of mucosal defence):

It contains surface epithelial cells with the intercellular junctions and secretes the bicarbonate and mucus. It produces heat shock proteins (HSPs) for maintaining of the cellular homeostasis during various oxidative cellular injuries. Prostaglandins (PGs) are formed likewise by the second line barrier for the repair of gastric epithelial integrity and protection of the gastric mucosa (Bobach et al., 2014). Epithelial integrity of stomach involves a specific balance between cell death and cell proliferation that controlled by epidermal growth factor (EGF) and transforming growth factor alpha (TGF-alpha) peptides. Furthermore, nitric oxide (NO), calcitonin gene-related peptide and some hormones comprising gastrin and cholecystokinin, ghrelin, leptin and gastrin-releasing peptide have been attributed in part to the release of PGs. This barrier yields trefoil factor family (TFF) peptides, and cathelicidins to inhibit the bacterial colonization and increase the intrinsic defensive system and ulcer healing in the stomach (Chen et al., 2015).
3. **Sub epithelial barrier (the third line of mucosal defence):**

It consists of nerves and blood flow that show an important role in the maintenance process of gastric integrity, mainly for providing oxygen and nutrients and eradicating toxic substances to ensure the normal tissue microcirculation. The maximal blood supply mediated to the regenerated epithelium by the sensory afferent nerves that produce calcitonin gene-related protein (CGRP). CGRP inducts endothelial cells to secrete prostacyclin and NO for vasodilatation and allows maximal blood supply to the regenerating epithelium in the stomach and remove the acid and toxins (Boltin & Niv, 2014).

Alterations in the gastric mucosal defence barriers and functions damage could be occurred through multiple factors. The imbalance between the mucosal defence and the offensive factors in the stomach would reduce the gastric integrity and enhance ulcer formation. The offensive physical, chemical or psychological factors are numerous. They can cause injury in the epithelial gastric barriers and induce ulceration in the stomach of human and experimental animals. Examples of these factors are gastric hydrochloric acid (HCL), *Helicobacter pylori* (*H. pylori*) infection, drugs consumption (non-steroidal anti-inflammatory drugs (NSAIDS)), proton pump inhibitors (PPIs) (omeprazole or histamine H2 receptor antagonists), alcohol intake, smoking, nutritional deficiencies, prolonged anxiety, psychological stress, surgical shock, burns and hereditary predisposition (Shaker *et al.*, 2010; Ji *et al.*, 2012). Previous studies of Sugano *et al.* (2012) and Shu *et al.* (2013) reported that the most common causes of gastric ulcer disease are the infection with *H. pylori* and ingestion of NSAIDs.

*H. pylori* infection remains an essential offensive factor that is related to the development of gastric and duodenal ulcers diseases, gastric cancers such as mucosa-associated lymphoid tissue (MALT) lymphoma and GIT disorders symptoms. *H. pylori*
is a gram-negative, unipolar, multi-flagellate, spiral shaped, microaerophilic bacterium that colonizes selectively at gastric mucosal tissue in the host. The fate of host cells can be determined by the interactions between the host cells and bacterial factors. The interactions are demonstrated through elevated of cell proliferation and elevated or reduced apoptosis of cells. Moreover, *H. pylori*-induced gastric epithelial cells apoptosis is often accompanying to reactive oxygen species (ROS) production. Therefore, the excessive induction of ROS production in gastric epithelial cells can damage Deoxyribonucleic acid (DNA) and cause gastric carcinogenesis (Handa *et al.*, 2010).

The second most offensive factor of stomach ulcer is the long-term consumption of NSAIDs for instance, ibuprofen, diclofenac, and aspirin. The NSAIDs are used for the chronic inflammatory diseases treatment is associated with the damage of gastric mucosa integrity, gastric bleeding, and decrease in inherent antioxidant defense of gastric mucosa, apoptosis of mucosal cells and inhibition of cell regeneration. Thus, they are inhibiting cyclooxygenase (COX), which is required for the creation of prostaglandins and leads to the increase in severity of gastric mucosal injury (Bindu *et al.*, 2013).

The etiology of gastric ulceration caused by alcohol is still not fully understood. However, the excessive ingestion of ethanol is serving as the highest incentive of acute gastric ulcer in humans and animals through increasing acid secretion, neutrophil infiltration, pro-inflammatory cytokines release, and increase of oxygen radicals. The alcohol-induced gastric mucosal injury can be modulated or mediated directly or indirectly in pathophysiology by several cellular molecules (Sowndhararajan & Kang, 2013) (Figure 2.3).
In the gastric ulcer, the propagation or initiation of oxidative chain reactions can occur through mediation of free radicals and agents of oxidative stress. The ROS free radical (one of an important oxygen radical) having high reactivity can change the cellular levels leading to cell death. It appeared by attacking the important cell constituents and leading to toxic compounds formation. Besides, ROS overproduction is implicated in the pathogenesis of a wide variety of disorders including inflammatory diseases, cancers, atherosclerosis, diabetes mellitus, malaria, neurodegenerative diseases and HIV/AIDS (Awah et al., 2010). It was stated that excessive ROS generation reduces various enzymatic and non-enzymatic antioxidants, for instance, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), endogenous glutathione (GSH) and sulfhydryl groups (NPSH) (Boligon et al., 2014). Moreover, superoxide anion radicals, OH· hydroxyl radicals and lipid peroxides are involved in tissue injury, lipid peroxidation and impairment of the gastric defensive factors of mucus and mucosa circulation. Therefore, a lot of therapeutic agents, either synthetic or natural, are used to reduce free radical pathologies and to improve human health (Ineu et al., 2013; Sidahmed et al., 2013).

Figure 2.3: Pathophysiology of alcohol-induced mucosal damage (Boltin & Niv, 2014).
2.1.3 Gastric ulcer treatment

Gastric ulcer treatment effectively depends on the accuracy of diagnosis and the effectiveness of medications in patients (Fashner & Gitu, 2015).

2.1.3.1 Gastric ulcer diagnosis

Usually, the diagnosis of gastric ulcer is based on the history, clinical features and specific testing of patients. Both of natural history and clinical features of gastric ulcer differ in individual populations. However, the main symptoms of gastric ulcer including burning epigastric pain that occurs 2-5 hrs after eating food or on an empty stomach and relieve the nocturnal pain by food intake, antacids, or anti-secretory agents. The vomiting, loss of appetite, dyspepsia intolerance of fatty foods, heartburn, and an affirmative family history are less common features. Weight loss and fear of food intake are characteristics of gastric ulcers evaluation. The patients should be evaluated for alarm symptoms. The alarm symptoms as anemia, hematemesis, melena, or hemepositive stool is indicator of bleeding; vomiting suggests obstruction; anorexia or weight loss proposes cancer; persisting upper abdominal pain radiating to the back indicates to the penetration; and severe, spreading upper abdominal pain suggests perforation. On the other hand, the diagnostic tests of gastric ulcer are mainly estimated using diagnosis of \textit{H. pylori} which are serological antibodies tests via serum enzyme-linked immunosorbent assay (ELISA), urea breath tests, stool monoclonal antigens tests, endoscopy with biopsy (Higashimaya \textit{et al.}, 2013; Fashner & Gitu, 2015).

2.1.3.2 Gastric ulcer drugs

Many people in the world take several remedies and drugs to support the mucosal defence mechanism in the stomach to prevent or treat the gastric ulcer. The healing of gastric ulcer is a spontaneous and complicated array of different mechanisms. These mechanisms work in tandem to restore the balance between the aggressive (acid, pepsin,
pro-inflammatory cytokines and *H. pylori*) and defensive factors (mucus, bicarbonate, blood flow and growth factors) in stomach. Gastric ulcers treatment involves the inflammation, the reconstruction of epithelial structures and the underlying connective tissue, the cell proliferation particularly at the edge of ulcer and angiogenesis development. Hence, the pharmacological modulations of molecular and cellular targets are necessary for the healing process and modification ulcer repair. These targets are either countering the offensive factors or stimulating the mucosal defensive factors to get rid of pain, heal the ulcer and delay ulcer relapse (Luo et al., 2015; Sperandio et al., 2015).

Currently, many medications have been used in the gastric ulcer therapy and depend on causative agents. Antacids are the easiest manner to reduce and neutralize the gastric acidity with weak bases in the stomach. The stomach secretes about 2–3 L of HCL and enzyme-containing gastric juice per day from the gastric glands. Generally, liquid preparations of antacids are more efficient than tablets that contain aluminium hydroxide, magnesium hydroxide, magnesium trisilicate, calcium salts or sodium bicarbonate in some mixtures. They enhance the protective prostaglandins production in the stomach and combine with noxious elements (Rozza et al., 2011).

Antibiotic medications have been prescribed and recommended also for killing *H. pylori* and some microbial agents for the stomach ulcer treatment such as amoxicillin, clarithromycin and metronidazole. Moreover, the greater risk of ulcer, the more virulent strains spread in the environment of stomach. Therefore, the principle of triple therapy of antibiotics (bismuth salt, metronidazole and either tetracycline or amoxicillin) is used currently for disruption and inhibition the mechanism of *H. pylori* colonization and for effectively bacterium eradication within the stomach environment (Silva & de Sousa, 2011).
Additionally, non-steroidal anti-inflammatory drugs (NSAIDs) are widespread used in the gastric ulcers and many diseases therapy due to their antipyretic, anti-inflammatory and analgesic activities. Based on their chemical structure, there are now at least 20 different NSAIDs used for humans such as aspirin, diclofenac, ibuprofen, indomethacin, diflunisal, meclofenamic acid and nabumetone (Silva & de Sousa, 2011; Sugano et al., 2012). Though, proton pump inhibitors (PPIs) such as omeprazole (the reference drug of this study), anticholinergics and histamine H2 receptor antagonists are often involved in the treatment of stomach ulcer through the suppressing Hydrogen potassium adenosine triphosphatase (H\textsuperscript{+}/K\textsuperscript{+}ATPase) and blocking the stomach acid secretions (Diniz et al., 2013).

Generally, the previous anti-ulcer medications caused specific structural and functional changes in stomach epithelial cells and numerous adverse unusual side effects, for instance; hypersensitivity, gynecomastia, impotence, arrhythmia and hematopoietic changes (Ji et al., 2012). Sometimes anti-acid drugs are unsuccessful as a result of drug interactions, recurrence after treatments and impair the absorption of acid gastric medium requirements for bioavailability (Abdelwahab et al., 2013).

Nowadays, the alternative medicine and the medicinal plants used to treat some ailments as wound and diabetic ulcers, heart disease, high blood pressure, pain and asthma. The medicinal plants can also treat and prevent the recurrence of gastric ulcers without many negative side effects (Hossain & Nagooru, 2011; Chen et al., 2012).

2.2 Wound healing

2.2.1 Wound

Wound is the oldest suffering common health problem among mankind. About 11 million people in the world are suffering from acute injuries and 0.3 million people are
hospitalized per year. Additionally, 6.5 million people are suffering from chronic wounds. It also has been reported about 1-2 % of the population will experience chronic wounds during their lifetimes in the developed countries (Wong et al., 2015; Mu et al., 2016).

The wound is defined as damage occurred through ruptured, cut or torn in the skin. The skin is the first important defensive line of the body against huge microorganisms and protects the body from water loss with thermo-regulative property. Wounds are more subjected to the infections and other troublesome complications either open wounds or closed wounds. They may be produced by physical, chemical, thermal, immunological or microbial insult to the skin tissue which is one of the most easily injured human organs (Miller et al., 2015).

2.2.2 Wound types

Mostly, the wounds can be categorized as open or closed wounds according to the underlying cause of wound formation and acute wounds or chronic wounds according to the basis of wound healing physiology.

The open wounds have a cracked open skin and the underlying tissues expose directly to the outside environment. Thus, in turn make it more vulnerable to infection and bleeding. They are further classified into incised, tear or lacerations, superficial or abrasions, puncture, avulsions penetration and gunshot wounds. However, the closed wounds have an intact skin and the underlying tissues expose indirectly to the outside environment. Despite, the skin is intact; the harm can reach down to the underlying tissues, internal organs, muscles and bones. Moreover, in some cases the blood escapes the circulatory system, remains inside the body and causes bruises or contusion, haematomas or blood tumour (Hajiaghaalipour et al., 2013; Agyare et al., 2016).
On the other hand, the acute wounds are defined as tissue injuries in the skin that normally proceed through timely and orderly reparative process. It leads to continued restoration of functional and anatomic integrity. Acute wound includes an incision wound, incomplete thickness damage and special tissue lack. It finalizes the wound healing in a well-organized process within the predictable time frame. The most frequently occurring are minor acute wounds, such as small cuts, abrasions and scraps. Therefore, the skin after damage requires the function maintenance by proper care to repair, prevent complications and avoid the risk improving to chronic state. Otherwise, chronic wounds are wounds that fail to progress through the stages of normal healing. Then they pass in a pathological inflammation state. This kind of wounds may be subclassified according to the aetiologies of chronic skin ulcers into diabetic, pressure and vascular ulcers (venous and arterial ulcers) (Gainza et al., 2015; Agyare et al., 2016). Chronic wound requires a prolonged time and extensive treatment to heal. Local infection, trauma, foreign bodies, hypoxia and systemic problems, such as diabetes mellitus, immunodeficiency, ischaemia, ageing, malnutrition or medications ageing are the most frequent causes in the chronic wounds. These often lead to delay of wound healing cascades. The wound healing process at the damaged site usually comprises numerous cell types, growth factors, cytokines, and extracellular matrix (ECM) components. Mostly, the healing is resulting of the extremely intricate process of scar formation in the skin (Delavary et al., 2011; Peng et al., 2012).

2.2.3 Wound healing process

Wound healing is a dynamic, intricate, and auto process of injured site skin involving biochemical, cellular, molecular, and physiological phenomena. These are resulted in the connective tissue repair and fibrous scar formation and lead to restoration of anatomical continuity and functional status of the skin. Healing of wounds starts from
the time of injury, and might proceed for different periods depending on the intensity of the wound in the skin (Kant et al., 2014).

The skin can be a defensive environment that is characterized by acidic pH, large dried regions, and continual shedding of superficial skin cells. Host skin defence comprises molecules such as lysozymes, proteases and antimicrobial peptides (Kong, 2011). Moreover, the skin is mainly divided to epidermal, dermal and hypodermal (subcutaneous fat) layers as shown in Figure 2.4. The epidermis is a barrier layer that mainly includes keratinocytes, basal stem cell and melanocyte whereas the dermal part is a structural support layer that comprises of fibroblasts (cellular compartment) and extracellular matrix (ECM) (acellular compartment). Furthermore, the dermis contains epidermal appendages, such as bulge stem cell, hair follicles, sebaceous glands, sweat glands and number of blood vascular assemblies to supply the skin and provide transport for nutrients, wastes, bioactive mediators, and immune cells within the skin. All compartments of epidermis have their own specific function to protect the body against pathogens and water loss. Therefore, the broken of protective barrier (skin) requires highly integrated and overlapping phases to be healed. The phases of wound healing process determine the potency and emergence of the healed tissue in the wounds. They are well controlled in the body through special mechanisms and any prolongation, deviation or problem in the regulation of this process can result in chronic wounds or delayed wound healing (Gouin & Kiecolt-Glaser, 2011; Dhiyaaldeen et al., 2014).
2.2.4 Wound healing phases

The wound healing process in the skin usually depends on itself to repair at the beginning of damage. It is well-coordinated biological and multifactorial sequence process that begins from the moment of injury and remains for unpredictable periods of time. The period is depending on the extent of the wound area and the health status of the injured individual (Agyare et al., 2016). Mainly, the process of skin healing is divided into four overlapped phases: coagulating (haemostasis) phase, inflammatory phase, proliferative phase (creation of granulation tissue and synthesis of collagen), and the remodelling phase, which eventually determines the appearance and strength of the healed tissue. They are well controlled in the body by special mechanisms that include the integrated interaction of different growth factors, cytokines, enzymes and cell types, such as inflammatory cells, keratinocytes, fibroblasts and endothelial cells (Gainza et al., 2015).

2.2.4.1 Coagulating (Haemostasis) phase

Skin injury produces cell damage and destruction in the blood vessels. The skin after wounding within seconds starts to avoid blood vessels constrict, blood loss by the activation of platelets aggregation, clotting and other accompaniment cascades. After

![Figure 2.4: The skin tissue structure (Miller et al., 2015).](image-url)
the ADP (adenosine diphosphate) leaking from damaged tissues, the platelets adhere to the exposed type 1 collagen and secrete adhesive glycoproteins for platelet aggregation.

Moreover, they secrete factors for interaction with and stimulation the intrinsic clotting cascade by thrombin production and fibrin formation. The haemostatic blood clot is a dynamic matrix of cells and proteins that serve as a protective shield temporarily for the wounds, act as a network for entering the inflammatory cells and provide a pool of cytokines/growth factors. It is mainly comprised of cross-linked fibrin, fibronectin, vitronectin, trombospondin, erythrocytes and platelets. Platelets are one of the initial sources of cytokines that mediate chemotaxis and macrophage activations (Delavary et al., 2011). The platelets also secrete the growth factors such as platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β), epidermal growth factor (EGF) and fibroblast growth factor (FGF) which act as chemo-attractants to phagocytic immune cells. There are also cytokines and chemokines can attract the monocytes to the wound bed such as ‘Regulated on activation normal T cell expressed and secreted’ (RANTES or CCL5), MCP-1 (CCL2), MIP-1 alpha (CCL3), TGF-alpha, fibronectin, elastin, C5a, C3a, nerve growth factor and ECM-components. These chemo-attractants have a vital role in the movements of several inflammatory cells, especially monocytes and neutrophils to wound site to initiate the inflammatory phase (Agyare et al., 2016).

2.2.4.2 Inflammation phase

The hemostasis and release of chemo-attractants can be manifest as the initiation of this phase. NO mediates the antiplatelet effects and vasodilation in this phase (Kang et al., 2015). In addition, the chemokines, vasodilation (mainly through histamine) and rise in blood vessel permeability can facilitate the recruitment of immune cells. Many of pro-inflammatory cytokines could be released from the new clot and directly from the injured tissues such as transforming growth factor (TGF-β), platelet-derived growth
factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) and Interleukin 8 (IL-8/CXCL-8) and act as potent chemotactic signals to instantly recruit neutrophils to the wound. This phase involves a well-characterised sequence of immune cell infiltration, neutrophils followed by macrophages then finally T-lymphocytes (Portou et al., 2015).

Neutrophils are the first inflammatory leukocytes that migrate to the inflammation area and clear the wound through two ways: cell mediated and immune-mediated pathways. They remove the microorganisms, dead cells remnants and toxic secreted substances such as ROS and proteases resulting in the lysis of the bacteria and debris. They are also considered as a major source of different pro-inflammatory cytokines such as IL-1alpha, IL-1beta, IL-6 and Tumor necrosis factor alpha (TNF-alpha) that stimulate the phagocytosis by macrophage (a differentiated monocyte) and release intracellular enzymes into the surrounding matrix. Once neutrophils are depleted, the second inflammatory cells (monocytes) will proceed to the wound for normal healing in response to chemotactic stimuli. Monocytes start binding to the endothelium and initiate the adhesion cascade by the selectin family (the cell-surface proteins of adhesion molecules) or by alpha 4-integrins. They bind also to specific proteins, like CAMs (cellular adhesion molecules) of the ECM (extracellular matrix). Moreover, several cytokines like IL-13, IL-10, IFN-y, IL-4 and ECM-components stimulate monocytes to differentiate into macrophages, which peak around 42 hrs after injury (Delavary et al., 2011; Akbik et al., 2014).

Macrophage involved in phagocytosis of the bacterial cells or the dead cells undergo apoptosis. Macrophages can survive without undergoing apoptosis and stay in the wound area to exert many functions that influence the healing process such as stimulation of collagen creation, angiogenesis and re-epithelialization. Macrophage M2
TGF-beta is a prominent source of TGF-beta, which promotes many aspects of wound healing. TGF-beta is an important cytokine and a multipotent protein, which influence fibroblast function, chemotaxis and ECM deposition. TGF-beta signals activate intracellular regulatory proteins known as Small mothers against decapentaplegic (Smads) that is an indicator of improving the wound healing and inhibition of fibrosis. Macrophages provide an ongoing source of pro-inflammatory cytokines, including IL-1beta, IL-1alpha, IL-6 and TNF-alpha, which are not only responsible for the control of inflammatory cell adhesion and migration, but are also important for the stimulation of fibroblast and keratinocyte proliferation. In this phase, the activated macrophages facilitate also the endothelial cells and smooth muscle cells proliferation (Macdonald et al. 2010).

T-lymphocytes are another type of inflammatory cells that reach lastly to the wound area based on macrophages signals. While their particular role is poorly understood, they perform differential roles of Cluster of differentiation 4 (CD4+) T helper and Cluster of differentiation 8 (CD8+) cytotoxic T cells, with CD4+cells found to have an affirmative promoting effect on healing, and CD8+cells an inhibitory effect. In addition, T-lymphocyte has regulatory effects on the inflammation and fibrosis. And the dermal subgroup of gamma delta T cells creates insulin-like growth factor 1 and keratinocyte growth factor (KGF) which stimulate proliferation of keratinocyte, supporting healing (Portou et al., 2015). Conversely, a delay in the migration of T-lymphocytes to the wound sites can produce extended and impaired healing process (Havran & Jameson, 2010).

2.2.4.3 Proliferation phase

The proliferation phase begins by macrophages after 4 days of injury and usually continues pending day 21 in the acute wounds, depending on the patient health and the
wound size. It involves angiogenesis (capillary growth), fibroblast proliferation (collagen deposition), granulation tissue (new blood vessels, fibroblasts, myofibroblasts, inflammatory cells, endothelial cells, and extracellular matrix) formation, wound contraction and re-epithelialisation. The fibroblasts and endothelial cells are the major key players of facilitating the previous involvements under control of the complex interplay of different growth factors such as FGF, TGF-β and VEGF, PDGF, fibroblast growth factor 2 (FGF2) and Insulin like growth factor 1 (IGF-1) (Abdul Latif et al., 2015; Agyare et al., 2016). The delayed wound contraction and reepithelialization are produced by the depletion of macrophages in the wound (Delavary et al., 2011).

2.2.4.4 Remodelling phase

This phase is the longest and last phase of the healing process in wounds and remains sometimes for several months to 2 years (Agyare et al., 2016). Remodelling phase is important for the scar strength and appearance. It mostly depends on breakdown of tissue and production of ECM and macrophages play an essential role in the two processes. The tissue breakdown is regulated directly and the ECM productions (especially collagen production) are regulated through fibroblasts. The main processes in this phase are the rearrangement, cross-linking of initially deposited collagen fibers and type III collagen substitution to type I collagen through the action of collagenases and matrix-metalloproteases (MMPs) (Portou et al., 2015).

During this stage, the proliferation of cells starts to slow down with reduction of protein synthesis and the collagen remolds into greater, more organised fibrils (type I collagen). The remodelling and realignment of collagen tissue occurs to create more tensile strength tissue comparable to that of normal skin. Moreover, cell and capillary density decrease the most of endothelial cells, macrophages and myofibroblasts to undergo apoptosis, or exit the wound. The last phase of normal wound healing showed
decreasing and changing of the macrophages numbers and activities that could result in less ECM production with contraction of the wound in the size and volume. The remodelling phase in the pathological conditions demonstrated a deviation in both numbers and functions of macrophages that could be produced in aberrant scarring (Delavary et al., 2011; Upadhyay et al., 2013).

2.2.5 Factors effects on wound healing

In the normal wound healing, the adequate haemostatic functions for the appropriate period take up to 4 weeks in the clean and germ-free wounds. The bleeding disorders including haemophilia B, deficiency of factor XIII and abnormalities of fibrinogen have revealed effects on normal wound healing. Therefore, normal healing requires adequate haemostatic function for the appropriate time frame (up to 4 weeks in the clean and uncontaminated wound). However, the impairment of one or more of the healing phases leads to non-healing wounds by several factors. The factors effects on wound healing can be divided into two main factors: (1) Intrinsic factors that are related to the wound itself, and (2) Extrinsic factors that are related to the patient. Many of them are inter-related factors and most of the extrinsic factors perform their functions through intrinsic effects (Teller & White, 2011; Rodriguez-Merchan, 2012).

Multiple factors might affect the healing comprising: diabetes mellitus, infection, ischemia and hypoxia, stress, poor nutrition and malnutrition, alcoholism, old age. The ischemia and infection are the most common factors resulting in impaired wound healing (Rodriguez-Merchan, 2012).

2.2.5.1 Diabetes mellitus

The impaired wound healing is one of complications of diabetes mellitus that resulted in chronic wounds with compromised microcirculation. Diabetes is now considered a widespread disease and is projected to increase approximately from 170
million to 365 million in 2030 worldwide (Tam et al., 2011). Inappropriate wound healing control might result in foot ulcers or even amputation. It is estimated that 15% to 25% of diabetic patients have foot ulcers and 3% will involve lower limb amputation (Ebaid et al., 2013).

Diabetes mellitus is associated with complex metabolic disorders including many organs and can devastate the lives of affected people. It is characterized by increasing levels of glucose in serum and deviations in carbohydrate, lipid and protein metabolisms that are initiated by modifications of insulin secretion, insulin action or both. The highest factors that are responsible for increasing the number of diabetic patients include the growth and aging of the population and also variations in lifestyle (Moura et al., 2013). Diabetes can be categorized into type I, type II and gestational diabetes. Diabetes type I, insulin-dependent diabetes mellitus (IDDM), or juvenile-onset diabetes, is caused by pancreatic β-cell damage, leading to absolute insulin deficiency and, subsequently, to the total requirement of exogenous insulin to sustain life (Vehik & Dabelea, 2011).

However, type II of diabetes mellitus is known as non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes, the type of diabetic models in the study, is characterized by insulin resistance. It might be combined with comparatively reduced insulin secretion levels. Type II diabetes affects approximately 90% of all diabetic patients and its main risk factors are increasing of glucose levels in serum in each fasting state or after food to be a sedentary lifestyle. The last type of diabetes mellitus is the gestational diabetes or impaired glucose intolerance, that is first diagnosed during pregnancy, is a carbohydrate intolerance during gestation. Gestational diabetes affects around 14% of pregnancies and is likewise a significant risk factor for type II diabetes in women. In addition, the obesity, coronary heart disease, stroke, diabetic nephropathy
and diabetic retinopathy are more likely developed in diabetic patients. Diabetic neuropathy and peripheral vascular diseases are usually the major factors involved in diabetic foot ulcers (DFUS) that is one of the most feared complications of diabetes. In the diabetic patients, the high concentration of glucose with uncontrolled diabetes has been associated with inhibition of the wound healing process (McCance, 2011; Moura et al., 2013).

The diabetic wound environment varies from the normal wound process through an extended and persistent inflammation phase with disruption of other healing phases that lead to slow repair in the wounds. In this disease, oedema and localized ischemia obstruct the delivery of oxygen and nutrients to the tissues and fail to reinstate the anatomical and functional integrity of the skin and persist for longer periods (Tam et al., 2011). The impaired healing that occurs in individuals with diabetes involves hypoxia, dysfunction in fibroblasts and epidermal cells, impaired angiogenesis and neovascularization, high levels of metalloproteases, damage from ROS and AGEs, decreased host immune resistance, and neuropathy (Guo, S., & DiPietro, L. A., 2010). The influence of these factors on wound healing is summarized in Fig. 2.5. The delayed collagen synthesis, impaired endothelial function, impaired epithelialization, defected angiogenesis, and diminished fibroblast proliferation have been perceived throughout the proliferation phase. Moreover, the fibroblasts do not create sufficient amounts of ECM and keratinocytes can not reepithelialize the skin that accompanied with increasing of apoptosis in the wound (Al-Bayaty & Abdulla, 2012; Bagdas et al., 2015).
The levels of growth factors in diabetic wounds have been verified to decrease and lacking blood perfusion coupled with impaired angiogenesis obscures tissue repair. Moreover, the high levels of glucose affect on leukocyte function. Therefore, the infiltration of macrophages and neutrophils is exaggerated and continuous, which is associated with deregulated and prolonged expression of chemokine such as MIP2 macrophage inflammatory protein 2 and MCP1 macrophage chemoattractant protein 1 (Chereddy et al., 2015). Therefore, elimination of microorganisms and wound debris has not proceeded regularly and release of cytotoxic enzymes at the local tissue damage in the host and generation of oxygen free radicals such as superoxides have increased. These can lead to reduce the effectivity of innate immune system. (Wong et al., 2015).

In addition, oxidative stress with excessive ROS production has been implicated in the pathology of diabetes mellitus. Therefore, exclusion of ROS with growth factor therapies is an essential strategy to induce angiogenesis and improve the wound healing in diabetic patients (George et al., 2014).
2.2.5.2 Infection

All wounds are virtually subjected to the infections by bacterial colonization. The microbial ecology of infected wounds is complex. However, the most common species isolated in both prospective and retrospective studies worldwide are Staphylococcus aureus and coagulase-negative staphylococci are the most common. There are other bacteria frequently related with wound infections such as β-haemolytic Streptococci, Escherichia coli, Pseudomonas aeruginosa, and Acinetobacter species (Wong et al., 2015).

The presence of bacterial colonization and proliferation in wounds induces a non-stop influx of polymorphonuclear leucocytes. It leads to release of cytotoxic enzymes, inflammatory mediators, oxygen free radicals and matrix metalloproteases in the host that produce wide local tissue damage (Guo & Dipietro, 2010).

The selected antibiotics for the controlling of wound infections are dependent on the results of culture and susceptibility tests and the microbes have many ways to adapt to antibiotic treatment. The inappropriate usage of antimicrobials may set patients at risk with significant adverse side effects due to the antimicrobial resistance. Therefore, the observation of antimicrobial resistance in wounds is required to monitor the effect of treatment (Wong et al., 2015).

2.2.5.3 Ischemia and hypoxia

Ischemia is an insufficient blood flow to the tissue that is considered a common factor in non-healing wounds. It causes hypoxia that is an insufficient oxygenation to the tissues. Temporary hypoxia after injury activates wound healing; however, prolonged or chronic hypoxia interrupts wound healing. Therefore, the insufficient oxygenation is a key element of healing failure and results in a failure of the adequate energy generation for normal healing (Azuma et al., 2012).
Otherwise, oxygen has a vital role in the cell metabolism mainly energy production. The oxygen concentrations affect the proliferation of fibroblast and the hydroxylation of lysine and proline during collagen synthesis. Without sufficient hydroxylation, collagen thermal stability is significantly impaired, leading to reduced tensile strength of wounds. Additionally, hypoxia can prompt the important promoters of cell proliferation, migration and chemotax through productions of growth factors and cytokine from keratinocytes, macrophages and fibroblasts including platelets derived growth factor, TGF-β, TNF-α, vascular endothelial growth factor and endothelin-1 (Zimmermann et al., 2014).

The only phase of wound healing that is stimulated through hypoxia is the angiogenesis induction. Nevertheless, appropriate maturation of new capillary networks involves a normal oxygen tissue pressure. Furthermore, hypoxia impairs the mechanism of body’s defence against bacterial attack because the oxygen is required for neutrophils to kill bacteria by creation of free oxygen radicals (superoxide) (Gouin & Kiecolt-Glaser, 2011).

Additionally, a heterodimeric transcription factor complex is known as hypoxia-inducible factor-1 (HIF-1) can mediate the cellular response to hypoxia. It has two subunits: a hypoxia-stabilized α-subunit (HIF-1α) and a constitutively expressed β-subunit (HIF-1β). HIF-1α is critical for the expression of multiple angiogenic growth factors, cell motility, and the recruitment of endothelial progenitor cell. Expression of HIF-1α has been induced in normal healing of wounds, while chronic wounds have low levels of HIF-1α. HIF-1α subunits translocate to the nucleus under hypoxic conditions then dimerize with HIF-1β, to allow binding the complex to the hypoxia response element (HRE) present in the regulatory sequences of many genes vital to cell survival (Hou et al., 2013; Zimmermann et al., 2014).
On the other hand, smoking disturbs oxygen partial pressures and produces more wound complications especially, after various types of surgeries. It has shown negative effects on cell viability, migration and myofibroblastic differentiation, immune function and collagen deposition (Sharif-Kashani et al., 2016).

### 2.2.5.4 Stress

Psychological stress generates the activation of hypothalamic-pituitary-adrenal and the sympathetic-adrenal-medullary axes. Higher glucocorticoids and catecholamines creation can directly influence many components of wound healing. Many studies on animal and humans have significant evidence that physiological stress responses can impede the initial inflammatory phase (Gouin & Kiecolt-Glaser, 2011). In chronic psychological stress, the patients have a systemically higher in the proinflammatory cytokines levels such as IL-1, IL-6, and TNF-alpha that have been associated with mental and physical health complications, involving depression and chronic inflammatory diseases (Koschwanez & Broadbent, 2011).

On the other hand, the psychological stress results in oxidative stress that occurs because of inequality between the ROS production and the protection through the cellular antioxidants. Increasing of ROS production and decreasing of antioxidant defense can cause tissue damage or even cell death that occurs by apoptosis and necrosis. ROS is enhanced under stress conditions such as low temperature, salt, drought, heat, oxidative stress and heavy metal toxicity (Thatoi et al., 2014). In addition, extreme ROS production produced the impaired wound healing. Moreover, the stress can modify the function of immune system that also might have adverse effects on healing process of wounds (Adly, 2010).

In the stress and high temperature, the heat shock proteins (HSPs), called heat stress proteins, are expressed and activated to protect the cells. HSPs are highly conserved
cellular proteins and found in all organisms such as yeast, bacteria, plants, animals and humans to protect the cells against the stress. HSPs have essential biological role in both normal and stress conditions. Normally, HSPs present as molecular chaperones in protein metabolism, cell cycle regulation, spermatogenesis and apoptosis and they maintain the cell integrity during normal cellular growth (Fang et al., 2012). Otherwise, the presence of stress stimuli in the organisms leads to higher significant expression of HSP, allowing organisms to resist these offenses and to maintain homeostasis (Vazzana et al., 2015). There are many stress stimuli such as changes in temperature, dissolved oxygen levels, osmotic pressure, heavy metals, and the presence of microbial infections. The HSPs synthesis and expression are regulated primarily via a transcription factor known as heat shock factor-1 (HSF-1) which binds to DNA at specific sites called the heat shock elements (HSEs) existing in the promoter region of specific genes. Based on the molecular weight of HSPs, their names can be modified such as HSP70 with 70 kilodaltons (kDa), HSP90 with 90 kDa, HSP100 with 100 kDa in dimension. This difference can produce different protective mechanisms in the cells against environmental stress (Han et al., 2016).

HSP70 protein is the most widely studied group of HSPs. It is highly homologous from bacteria to humans. It has essential extracellular functions for instance, activation of immune system and anticancer and has important roles in the folding and translocation of proteins, spermatogenesis, and in gastric and wound healing (Rajeshkumar et al., 2013).

2.2.5.5 Poor nutrition and malnutrition

Poor nutrition and malnutrition are known to both increase the risk of chronic wounds and delay or impair wound healing. Malnutrition is crucial that at-risk patient is identified at the early stages using the indication of based nutritional screening tool.
Malnutrition is a nutritional state in which a deficiency or excess (or imbalance) of energy, proteins and other nutrients produces a measurable adverse effect on tissue/body form, body function, and clinical outcomes. The basic metabolic rate (the amount of energy depleted at rest) increases after the individual has a wound because of greatly enlarged cell activity at the wound site. It rises up to 10% after minor surgery and 100% or more in the severe burns (Ellinger, 2015; Rabess, 2015). Gandy, (2014) reported that the Manual of Dietetic Practice identified the reasons of malnutrition into four reasons:

1. Impairment of intake.
2. Alteration of nutrient requirements.
3. Impairment of digestion and/or absorption
4. Increasing of nutrient loss.

Nutrition shows an important role in preserving tissue viability and in supporting tissue repair in the event of injury that is essential for wound care management. Optimum nutrition is a key element to maintain all phases of wound healing process. Each specific nutrient has specific role in tissue viability and wound healing (Posthauer et al., 2015).

Carbohydrate, especially glucose, is the primary energy source required for new tissues, wound healing and prevent the depletion of protein. Malnutrition of protein energy has been verified to have a direct influence on non-healing of wounds resulting in impaired collagen synthesis and deposition. Additionally, micronutrients play a vital role in wound healing, mostly the vitamins A, B1, B2, B6, C and E. These vitamins are antioxidants and have a metabolic function in energy production and collagen deposition. Vitamins, copper, zinc and selenium have immune function and their deficiencies lead to delayed wound healing and susceptible to infection. Zinc also has a role in all stages of wound healing and zinc excess can cause toxicity, induce both
copper and iron deficiency anaemia, which can eventually result in reduced oxygen delivery in the blood to the wound (Guo & DiPietro, 2010).

Furthermore, adequate healthy fluids are necessary for sustaining good skin tone and blood flow to tissues and preventing the dehydration that produces more and fast skin breakdown. Therefore, the minimum fluid intake required in the majority of the adult population is 1500 mL/day (the equivalent of 6-8 cups) however; this amount has been adjusted depending on the patient's clinical condition (Rabess, 2015).

On the other hand, alcoholism can produce a delayed wound healing through impairing the early inflammatory response, inhibiting wound closure, angiogenesis, collagen production, and changing the protease balance at the injured site. Clinical indications and animal experiments have revealed that drinking of alcohol increase the incidence of infection in the wound. The extreme effects of drinking the alcohol on host-defense mechanisms are dependent upon the pattern of alcohol exposure. The ethanol-mediated decrease in wound vascularity causes increased wound hypoxia and oxidative stress. The wound angiogenesis is decreased through up to 61% following a single ethanol exposure (Guo & DiPietro, 2010).

Hence, intake of a well-balanced and healthy diet and maintaining a stable healthy weight are imperative and can decrease the risk of developing several disorders such as diabetes, obesity and heart ailments that could predispose their patients to wounds and ulcers (Rabess, 2015).

2.2.5.6 Medications

The medications that interfere with the inflammatory responses and cell proliferation or clot formation or platelet function have a significant impact on healing. They include glucocorticoid steroids, NSAID drugs such as ibuprofen and aspirin, and
chemotherapeutic drugs such as adriamycin. Glucocorticoids can obstruct production of HIF-1, a key transcriptional factor in wound healing. However, NSAID drugs have demonstrated anti-proliferative effects and reduce the collagen synthesis, platelet aggregation and then impairing the coagulation phase. Chemotherapeutic drugs result in reduced cellular metabolism and impaired wound healing (Xie et al., 2011). Otherwise, chemotherapeutic drugs can inhibit the synthesis of DNA, RNA, or protein; cause decreased fibroplasia and neovascularization of wounds (Guo & DiPietro, 2010). The immunosuppression drugs of HIV, cancer and malnutrition patients as well showed impair the inflammatory response in wound healing cascades (Harper et al., 2014).

2.2.5.7 Old age

Old-age patients have slower inflammatory, migratory and proliferation responses, thinner epidermal layer in the skin and slower wound healing and they become more subjected to injuries as well as chronic diseases. With old age people, the functions of skin deteriorate due to changes of skin morphology and structure, influenced by intrinsic factors such as the genetic make-up, and extrinsic factors such as sun exposure and tobacco smoking. Additionally, aging is associated to a decline in systemic and local hormone levels. It is more obvious in postmenopausal women. Estrogens and androgens hormones and their steroid precursor dehydroepiandrosterone have substantial effects on cutaneous wound healing. Although androgens have negative regulatory effects on repair, the estrogens and dehydroepiandrosterone have positive regulatory effects on wound healing by reducing inflammation, and promoting ECM deposition. In addition, gene expression is different in the elderly adults compared with that in younger adults, and this variation is affected by estrogen and the elderly man has poorer wound healing compared with elderly woman (Kapetanaki et al., 2013; Sgonc & Gruber, 2013).
2.2.5.8 Genetics

Many studies have been motivated to both the cellular and genetic players in wound healing to investigate more about impaired wound healing. Genes can be a contributor to chronic non-healing wounds. For example, there is a strong genetic component in keloid scars development, being considerably more commonly in black, Hispanic or Asian race patients. Keloid scars occur when there is overgrowth of scar tissue that prolongs beyond the wound edges. The keloid scars are painful and itchy and have a high recurrence rate though can respond to steroids, cryotherapy or radiation therapy.

The incisional herniae have similarly been exposed to have a genetic component that causes a defect in collagen deposition, with higher levels of type III collagen accompanying with hernia development (Harper et al., 2014).

Moreover, the gene expression of collagen is under effect of the lipid peroxidation and/ or acetaldehyde formation that can be enhanced by gene expression of human collagen’s fibroblast cultivation (Zarandi et al., 2015). Collagen type I is commonly expressed in all vertebrates and generated predominantly by mesenchymal cells, such as fibroblasts, osteoblasts and smooth muscle cells. It is considered a critical determinant of organ formation, growth and homeostasis as result of being the most abundant component of the ECM. Structural and metabolic deficiencies of the collagen type I chains give rise to a large variety of heritable and acquired disorders of the connective tissue and fibrotic diseases, for instance, scleroderma, liver cirrhosis, glomerulosclerosis and idiopathic pulmonary fibrosis that severely impair organ function. Collagen type I is heterodimer and has two alpha 1 chains and one alpha 2 chain forming a triple helix structure. Structural integrity and coordinated biosynthesis of the two chains are critically essential for morphogenesis, growth and tissue homeostasis and healing.

Different diseases related genomic variants of type I collagen have been identified from single-base mutations of type I collagen alpha 1 chain (Col1a1) and type I collagen
alpha 2 chain (Col1a2) genes (Xu et al., 2016). It has been relied on using Col1a2 upregulation by some studies in transfected cells as a sensitive read-out to assess the efficacy of pharmacological inhibitors of fibrogenesis. The Col1a2 transgene has been widely used in experimental model to study the molecular mechanisms responsible for collagen I biosynthesis during development and in fibrotic conditions (Ponticos et al., 2004).

Martin & Nunan, (2015) reported that the studies of mouse wound transcriptome have shown many upregulated genes after damage, and some of these gene inductions occur in the epithelium of wound edges. The initial gene upregulations are classic immediate early genes, comprising Ap1, Fos and Jun and the krox zinc finger transcription factors. These presumably function as part of the transcriptional activation machinery for the several hundred genes that are upregulated in the cells in normal healing. Therefore, the late-activated genes, such as epidermal growth factor receptor, are commonly kept silent through histone methylation marks deposited via the polycomb family of epigenetic regulators, but the polycombs are downregulated and these marks are removed soon after wounding.

### 2.2.6 Apoptosis in wound healing

Wound healing includes sequences of rapid proliferations in specific cell populations that prepare the wound for repair, deposit new matrices then mature the wound. At the same time, these specific cells such as inflammatory cells must be eradicated from wound before the progression to the next phase of wound healing to have a balanced cell growth. Apoptosis is the physiological process of cellular down-regulation that allows to the removals of the inflammatory cells and inhibiting scar formation without tissue damage or inflammatory responses and avoids the pathological tissue repair. This process is considered as a highly conserved programmed cell death with genetically
control that maintains the appropriate cellular configuration of each tissue. It is resulting in chromatin condensation and membrane blebbing, cell shrinkage and cell disassembly for apoptotic bodies formation. Apoptosis has four main steps: inductions, detections, effectors, removals by phagocytosis and each step require the concerted effort of many molecules. The most effective molecules are cysteine-dependent aspartate-directed proteases (caspases), Bcl-2 family proteins and genome p53 (Laulier & Lopez, 2012; Jiang et al., 2014).

Caspase is a key effector molecule that starts the apoptosis to cause the morphological and biochemical variations. In addition, the p53 protein as well as Bcl-2 family proteins regulate apoptosis through transcription-dependent or independent mechanisms (Küpper et al., 2014; Huang et al., 2016). P53 protein can monitor the genome integrity and activate at least thirty different genes, which inhibit cell cycle and involved in apoptosis and in DNA-repair systems. It is functionally closely associated to Bcl-2 family proteins. P53 proteins result in enhanced expression of the Bax gene that initiates apoptosis and inhibits the creation of suppressing proteins of apoptosis, including Bcl-2. The Bcl-2 family has a large group of anti-apoptotic and pro-apoptotic proteins include Bax, Bak, Bcl-xL, Mcl-1, Bcl-W, Bcl-Xs, Bad, Bik, Hrk and Bid proteins. The disturbances in the balance among apoptosis-inhibiting proteins and apoptosis-inducing proteins produce dysregulation of the apoptotic processes. Subsequently, the unbalanced between the apoptosis and cellular proliferation produce abnormal healing in the wound, such as keloid formation and hypertrophic scars or might delay wound healing (Gryko et al., 2012; Laulier & Lopez, 2012).

2.2.7 Wounds Treatment

The initial management care of wounds is the basic requirements in all wounds treatment especially open wounds to be closed or healed. It incorporates the aseptic
technique and gentle tissue handlings and starts instantly after wounding by covering
the wound with a clean and dry bandage to avoid further contamination and reduce
haemorrhage (Davidson, 2015). Moreover, the systemic factors for instance, nutritional
and immunological status, stress, smoking and other medical diseases or disorders such
as renal failure, pressure ulcers and diabetes must be managed effectively for optimal
wound healing. Hence the majority in the treatment of wound heals is being without
complications and in a timely manner (Vowden & Vowden, 2014).

2.2.7.1 Wound Diagnosis

Wound treatment is based on the outcomes of diagnostic investigations and basic
approaches. They include vascular, neurological and microbiological diagnostics
examined through more specific investigations that depend on the suspected diagnosis
(Erfurt-Berge & Renner, 2015).

2.2.7.2 Wound Drugs

Many pharmaceutical agents used in wound management and treatment. They
include ointments, powders, creams, gels and solutions to apply on the wound site
(Vowden & Vowden, 2014). Furthermore, the growth factors have a potential
application in healing of wound such as epithelia growth factor, platelet derived growth
factor, fibroblasts growth factor, transforming growth factor, insulin-like growth factor,
human growth hormone and granulocyte-macrophage colony-stimulating factor
(Zarandi et al., 2015).

On the other hand, the wounds of burns, split skin, graft donor sites and diabetic
ulcers can lead to prolonged wound healing therefore they commonly require treatment
with antibiotics such as gentamicin and ciprofloxacin. The antibiotics have some side
effects such as nausea, diarrhea, dizziness, light-headedness, headache, or trouble
sleeping. Thus, accelerating the wound healing through local delivery of drug is
considered one of the effective ways in treatment that have essential swelling capability for absorbing excess exudates and oxygen permeability for respiring (Kang et al., 2010). There are various wound healing or dressing materials are being used for wound dressing from time for instance, honey, linen, animal fats and vegetables fibers and some herbs. The other significant active compounds in wound healing are vitamins A, C, E, zinc, and copper minerals. One of the main benefits of using bioactive wound dressing materials is the modified chemical environment facing the physiological conditions of wound for earlier healing that is more successful in clinical results (Kataria et al., 2014; Zarandi et al., 2015).

2.3 Free radicals and antioxidants

Free radical is a chemical reactive molecule (or molecular fragments) with one or two unpaired electrons in its outermost layer, which sometimes has half-life only few nanoseconds. This main reactive molecule can include additional, non-radical oxidative substances such as hydrogen peroxide (H$_2$O$_2$) or dinitrogen trioxide (N$_2$O$_3$). The main reactive molecules are reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are the most important free radicals with unpaired electron. Their derivatives are shown in the Figure 2.6 as below adapted (Wojtunik-Kulesza et al., 2016).

![Figure 2.6: The derivatives of the main reactive molecules of free radicals (Wojtunik-Kulesza et al., 2016).](image-url)
Free radical can be generated in multiple ways either exogenic or endogenic depending on the source of free radical. The endogenic free radical is produced from immune cell activation, inflammation, mental stress, excessive exercise, ischemia, infection, cancer, aging, neurodegenerative diseases, diabetes mellitus, and cardiovascular diseases. Nonetheless the exogenic free radical created from environmental effect as water and air pollution, industrial solvents, transition or heavy metals (cadmium (Cd), mercury (Hg), lead (Pb), Iron (Fe)), certain medications (tacrolimus, gentamycin, bleomycin, cyclosporine), cooking (smoked meat, used oil, fat), tobacco and radiations (Ergin et al., 2013; Barhé & Tchouya, 2014).

In biological systems, most free radicals are produced in physiologic or pathologic conditions in the body. Their roles could be constructive when they serve as signaling and regulatory molecules in physiologic conditions, but they could be destructive and cytotoxic oxidants in pathologic conditions. Therefore, protecting the living organisms involves the redox regulation process to regulate and maintain the balance between constructive and destructive effects of free radicals in the organisms (Sindhi et al., 2013).

The constructive effects of free radicals involve their roles in the body’s defence in contradiction of disease. For instance, the phagocytes in the immune system use the oxidative free radicals for destroying viruses and bacteria. They have also an essential beneficial role in some mechanisms of apoptosis of defective cells (Forman et al., 2014). Additionally, the formation of free radicals (ROS and RNS species) can be resulted by the enzymatic and non-enzymatic reactions in the cell. In mitochondria, the major source of free radicals, the non-enzymatic reactions of generating free radicals include the oxidative phosphorylation to generate ATP and produce the superoxide
radicals. On the other hand, the enzymatic reactions of generating free radicals involved in the respiratory chains, the phagocytosis, the prostaglandin synthesis and the cytochrome P450 system. On demand, most cells can create superoxide, hydrogen peroxide and nitric oxide, which indicates constructive role of free radicals in some processes in the organism (Awah et al., 2010; Tegeli et al., 2014).

Otherwise, the free radicals appear extremely to be the result of many different human disorders and diseases when they are overproduced. The excess production of ROS/RNS on one side and a deficiency of enzymatic and non-enzymatic antioxidants on the other appeared more in the harmful effects (Trifunovic et al., 2015).

The destructive effects of free radicals resulting in a potential biological damage is termed as nitrosative stress and oxidative stress depending on the side of molecule. The nitrosative stress is produced by overproduction of RNS that lead to nitrosylation chain reactions, alter the structure of cellular proteins, and inhibit their function. On the other hand, the oxidative stress is due to overproduction of ROS (Thatoi et al., 2014). The oxidative stress represents disorders in the equilibrium status of pro-oxidant/antioxidant reactions that use oxygen in the living cells. It causes numerous diseases, including diabetes, Alzheimer’s disease, atherosclerosis, Parkinson’s disease, glaucoma and age-related macular degeneration (Wojtunik-Kulesza et al., 2016). Hence, the excess formation of free radicals molecules revealed oxidant properties. They can oxidize many important compounds in the organism such as aldehydes, amino acids, unsaturated fatty acids then change the cell structure or other parts in the cell through interacting with biomolecules such as lipid, protein and nucleic acid. In addition, each molecule is characterized by a different reactivity and selectivity. For instance, the excess production of ROS, the most reactive molecule, leads to damage in the cellular lipids, proteins, or DNA inhibiting their normal functions. Moreover, the permanent
modification of genetic materials causing from oxidative damage represents the first stage involved in mutagenesis, carcinogenesis, and ageing (Lushchak, 2014; Zheng et al., 2014).

The cellular and subcellular lipids are greatly sensitive to free radicals and oxidative stress and can undergo very destructive chain reactions of lipid peroxidation under enzymatic control or non-enzymatic control. Lipid peroxidation produces variations in the biophysical properties of the membrane and inactivation of membrane-bound enzymes or receptors that may impair the normal cellular functions. It leads to generate peroxides and hydro-peroxides that can disintegrate to cytotoxic products most of which are aldehydes (Zheng et al., 2014). For example, malondialdehyde (MDA) that is the major aldehyde product of lipid peroxidation and is mutagenic in bacterial and mammalian cells and carcinogenic in rats and 4-hydroxy- 2-nonenal 4- that is a weakly mutagenic aldehyde and lipid peroxidation end product produced in the skin as a result of oxidative stress and can disrupt cellular metabolic activity. In addition, the high levels of definite oxidative damage biomarkers such as MDA seem to correlate with greater risk of disease in several clinical studies (Winczura et al., 2012; Roy et al., 2014).

This free radical mediated oxidative stress effects are results of poor natural antioxidant defense in the body. Therefore, decomposing of peroxides and/or binding with pro-oxidant metal ion and scavenging of free radicals are necessary in the organism for having free radicals in the equilibrium state. The equilibrium free radicals state usually appeared in the body by endogenous antioxidant defense exogenously administered antioxidants mechanism (Roy et al., 2014). Similarly, lipid peroxidation can be terminated by the reaction of lipid radical with a molecule of antioxidant such as
vitamin E that can create more stable radical and be recycled by other cellular antioxidants such as vitamin C or glutathione (Thatoi et al., 2014).

Presently, antioxidants have increased significantly because of their progressive involvement as health promoters in disorders and diseases for example cardiovascular problems, atherosclerosis, ageing, wound impairment, gastric ulcers and cancer. In human body, free radicals were scavenged through antioxidant enzymes and small molecule antioxidants (Roy et al., 2014).

Antioxidants or free radical scavengers are molecules that can minimize and prevent the oxidation of molecule by interacting with free radicals to break the chain reaction before vital molecules damage. These scavengers can perform at different levels of prevention, interception and repair. In addition, they can reduce the lipid component oxidation in cell membrane or inhibit the volatile organic compounds and the conjugated hydroperoxides which are carcinogenic components (Qader et al., 2011).

Antioxidants can be naturally generated (enzymatic or non-enzymatic endogenous antioxidants) or externally provided by foods (exogenous antioxidants). Human makes various antioxidants themselves to detoxify free radicals such as being SOD, CAT, GPx and glutathione reductase (GR) (enzymatic antioxidants). Moreover, other antioxidants can be obtained from the diet (non-enzymatic antioxidants) either actual for instance tocopherol (vitamin E); ascorbate (Vitamin C) or putative for instance flavonoids; carotenoids (Guo & DiPietro, 2010). Under normal conditions, the balance between both the activities and the intracellular levels of the antioxidants is essential for the survival of organism and its health. The Internal body source of antioxidants regulates the free radicals amount and supported via other external substances provided by food or supplements. The provision of antioxidants through
food is a simple means to prevent or reduce the development of illnesses. All nutrients, including carbohydrates, fatty acids, proteins, vitamins, and minerals, are necessary for cell metabolism. The nutrient antioxidant deficiency is one of the origins of numerous chronic and degenerative disorders. Each nutrient antioxidant has unique structure and antioxidant function (Zheng et al., 2014; Trifunovic et al., 2015).

Besides, Carlsen et al., (2010) reported total antioxidant capacity of more than 3100 fruits, vegetables and herbs in addition to common everyday foods examined over a period of eight years. Many antioxidants occur naturally occurring in the plants (Halliwell, 2012). The plant produces phytochemicals to protect itself, although it usually exhibits a variety of human health effects. Among the most structurally diversified phytochemicals are phenolic compounds which have attracted most attention for the diverse bioactivities that are vital in the reproduction and growth of plants and contribute to the color of fruits, leaves and flowers. They act as reducing agents;

phenolic acids, flavonoids, tannins, curcuminoids, coumarins, lignans, quinines and other phenylethanoids and phenylpropanoids. The largest group of phenolic compounds is flavonoids that have more than 4000 compounds isolated till now. They can be classified into a variety of classes for example flavones, flavonols, flavanones, flavanonols, isoflavones, flavan-3-ols and oligomeric flavonoids such as proanthocyanidins and tannins. In addition, vitamin C, vitamin E, selenium, β-carotene and other carotenoids (α-carotene, β-cryptoxanthin, lutein, lycopene, and zeaxanthin) are among the most regularly been considered as nutrient antioxidants (Trifunovic et al., 2015).

Therefore, there are intensive studies on natural antioxidants derived from plants to substitute the synthetic antioxidants. The use of natural products derived from plants
and herbs has been proposed as adjuvant therapy of diseases for many years (Mancuso, 2015).

2.4 Medicinal plants

2.4.1 The usage of medicinal plants

People have been used the medicinal plants (the most and the oldest widespread medications) for at least 5,000 years. Plants have an excessive potential for producing new drugs of great benefit to the humankind. They have being consuming as a preventative medicine in the traditional medicine and some of medicinal plants are utilized for treatment some ailments (Renisheya et al., 2011).

The medicinal plants have various parts and different medicinal properties such as antipyretic, antiulcer antiviral, anti-inflammatory hepatoprotective, anti-diabetic, antiangiogenic, anticancer, antibacterial and immunomodulatory activities (Qader et al., 2011; Antonisamy et al., 2014). Plants consist of minerals, vitamins, volatile oils, glycosides, alkaloids, bioflavonoids, and other constituents for supporting a particular herb’s medicinal properties. Rather than using a whole plant as different organic extracts, pharmacologist identifies, isolates, extracts, and synthesizes distinct components, then capturing the active properties and components. There are 121 chemical substances of known structure are still extracted from plants that are useful as drugs around the world. The chemical components derived from the plants remain the origin for a large proportion of the most medications used currently for the treatment of heart disease, high blood pressure, pain, asthma, and other problems. Medicinal plants can also assist in ulcer healing and in preventing recurrence (Hossain & Nagooru, 2011; Abdelwahab et al., 2013). Furthermore, they have natural antioxidant capacity which lead their consumptions to best health (Li et al., 2013). Hence, there are intensive
studies on natural antioxidants derived from plants to replace the synthetic antioxidants (Trifunovic et al., 2015).

2.4.2 The selected Malaysian plants

Malaysian plants have been widely used because of their valued aromas and tastes for foodstuffs which are used traditionally for the treatment of different human ailments such as Bauhinia thonningii, Polygonum minus, Andrographis paniculata, Curcuma xanthorrhiza, Momordica charantia and Strobilanthes crispus. The leaves and roots of many medicinal herbs have been used for various medicinal purposes in Asia and Europe. Many researchers have reported that many medicinal plants used in traditional folkloric medicines as antiulcer agents in the stomach and skin (Al Bayaty et al., 2010; Qader et al., 2011; Abdelwahab et al., 2013). The leaves of two Malaysian plants, C. barometz and V. pubescens, were selected in this study to evaluate their bioactivities and abilities toward gastro protection, wound healing potential. In addition, the plant extracts will also be examined for potential toxicity that is considered an essential step in estimating their suitability for application safely in folk medicine.

2.4.3 Cibotium barometz

2.4.3.1 Taxonomy of C. barometz

Cibotium barometz (L.) J. Sim. (family Dicksoniaceae) is known as”Jinmao Gouji” (Golden Hair Dog Fern) or penawar jambi (in the Peninsula of Malaysia) (Ong, H., & Nordiana, M. (1999). It is a tropical and subtropical fern native plant of China and South East Asia to the Malay Peninsula (Bobach et al., 2014). The taxonomy of C. barometz and its leaves are displayed in the following Table 2.1 and Figure 2.7.
2.4.3.2 Ethnopharmacological uses of C. barometz

Lai & Lim, (2011) stated that the ethnomedicinal uses of C. barometz are for the treatment of typhoid, dyspepsia and coughs, tonic for kidney and liver. However, the hair of C. barometz is applied on cuts and wounds to stop bleeding (Wu & Yang, 2009). Moreover, its rhizome is anti-inflammatory and an anodyne plant and is used to treat rheumatism, polyuria, leucorrhoea, menstruation problems, herniated disc, hyperosteogeny and osteoporosis (Zhao et al., 2011; Bobach et al., 2014). There are many phenolic compounds in Cibotium that showed potent antioxidant activities and
strong chelating power. *C. barometz* leaves extract has potential for further development as a natural alternative for the management of postmenopausal osteoporosis (Cuong *et al.*, 2009). Previous studies showed that *C. barometz* has antioxidative tyrosinase inhibiting and antibacterial activities (Cuong *et al.*, 2009; Zhao *et al.*, 2011). Furthermore, the dried rhizomes that are taken orally can be used as an anti-hepatitis virus agent and demonstrated hepatoprotective activities on tacrine-induced cytotoxicity in human liver-derived Hep G2 cells (Wu & Yang, 2009). This plant inhibits the formation of osteoclast without affecting on bone marrow-derived macrophage (BMM) cell viability (Wu & Yang, 2009). It is reported by Syafni *et al.* (2012) that 3, 4-dihydroxybenzoic acid and 3, 4-dihydroxybenzaldehyde in this fern were responsible for exhibited antimicrobial properties of this plant. This type of medicinal plant that applied in natural remedies is used in traditional healing and stop bleeding. The rhizomes comprise approximately 30% starch and β-sitosterol, caffeic acid, daucosterol, alternariol, (3R)-des-O-methyl lasiodiplodin, protocatechuic aldehyde, (24R)-stigmast-4-ene-3-one, 24-methyleneoctanol, 5-hydroxymethyl-2-furancarboxaldehyde, onitin, protocatechuic acid, N-butyl-β-D fructopyranoside, palmitic acid, 1-monopalmitin, and D-glucose as shown in Table 2.2 (Wu & Yang, 2009).
Table 2.2 Chemical compounds from *C. barometz* rhizome and their biological activities.

<table>
<thead>
<tr>
<th>Name of compounds &amp; Bioactivities (C. barometz rhizome)</th>
<th>Structure of compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Starch</strong>&lt;br&gt;The major source of energy in the diet of most human populations (Tovar et al., 2014).</td>
<td><img src="image" alt="Starch structure" /></td>
</tr>
<tr>
<td><strong>B-Sitosterol</strong>&lt;br&gt;Anti-inflammatory, antioxidant, antidiabetic, anticancer, antimutagenic (Saeidnia et al., 2014).</td>
<td><img src="image" alt="B-Sitosterol structure" /></td>
</tr>
<tr>
<td><strong>Daucosterol</strong>&lt;br&gt;Antioxidant (Zhang et al., 2014).</td>
<td><img src="image" alt="Daucosterol structure" /></td>
</tr>
<tr>
<td><strong>Onitin</strong>&lt;br&gt;Antioxidant and anti-inflammatory (Badole et al., 2014).</td>
<td><img src="image" alt="Onitin structure" /></td>
</tr>
<tr>
<td><strong>Alternariol</strong>&lt;br&gt;Antiviral, antitumor and anti-inflammatory (Lou et al., 2016).</td>
<td><img src="image" alt="Alternariol structure" /></td>
</tr>
<tr>
<td><strong>(3R)-Des-O-methyl lasiodiplodin</strong>&lt;br&gt;Antioxidant and anti-inflammatory (An et al., 2016).</td>
<td><img src="image" alt="Des-O-methyl lasiodiplodin structure" /></td>
</tr>
<tr>
<td><strong>Protocatechuicaldehyde</strong>&lt;br&gt;Antioxidant (Wu &amp; Yang, 2009)</td>
<td><img src="image" alt="Protocatechuicaldehyde structure" /></td>
</tr>
<tr>
<td>Compound</td>
<td>Description</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>(24R)-Stigmast-4-ene-3-one</td>
<td>Anticancer, anti-fungal and anti-inflammatory. (Wu et al., 2007; Kulip et al., 2015)</td>
</tr>
<tr>
<td>24-Methylenecycloartanol</td>
<td>Antioxidant, antihypertensive, antidiabetic, gastro protective, anti-inflammatory, neuroprotective activities (Hashmi et al., 2015; Wedler et al., 2015).</td>
</tr>
<tr>
<td>5-Hydroxymethyl-2-Furancarboxaldehyde</td>
<td>Antioxidant and antidiabetic (Loizzo et al., 2014).</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>Antioxidant (Wu &amp; Yang, 2009)</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>Antioxidant and antihepatic (Khadem et al., 2010)</td>
</tr>
<tr>
<td>N-butyl-β-D-Fructopyranoside</td>
<td>Anticancer (Lu et al., 2014)</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>Antioxidant and anticholinesterase (Öztürk et al., 2014)</td>
</tr>
<tr>
<td>1-Monopalmitin</td>
<td>Anti-inflammatory (Ding et al., 2013)</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>A source of energy (Wu &amp; Yang, 2009).</td>
</tr>
</tbody>
</table>
2.4.4 *Vitex pubescens*

2.4.4.1 Taxonomy of *Vitex pubescens*

*Vitex pubescens* Vahl (family Verbenaceae), is known as laban or halban in the Peninsula of Malaysia. Several varieties of *Vitex* have been found in distant countries in Indonesia, Brunei, India and Mexico (Ong, H., & Nordiana, M. (1999). *Vitex pubescens* has another used name that is *Vitex pinnata* as Ng, 1989 reported. The taxonomy of this plant and its leaves were shown in Table 2.3 and Figure 2.8.

**Table 2.3:** The taxonomy of *Vitex pubescens* leaves

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Division</td>
<td>Magnoliophyta</td>
</tr>
<tr>
<td>Class</td>
<td>Magnoliopsida</td>
</tr>
<tr>
<td>Order</td>
<td>Lamiales</td>
</tr>
<tr>
<td>Family</td>
<td>Verbenaceae/Lamiaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Vitex</td>
</tr>
<tr>
<td>Species</td>
<td><em>Vitex pubescens</em> Val.</td>
</tr>
</tbody>
</table>

**Figure 2.8:** The leaves of *Vitex pubescens* (Rimba Ilmu Botanic Garden, UM)
2.4.4.2 Ethnopharmacological uses of *Vitex pubescens*

*V. pubescens* is a medicinal herb used as folk remedies to treat various gastrointestinal diseases such as diarrhea, stomachache and ulcers and the paste of leaves is applied on the wounds and also used for the scorpion stings (Ong and Nordiana, 1999; Meena *et al.*, 2011). Batubatra *et al.* (2009) indicated that *Vitex* is effective for management of fever and antipyretic. *Vitex* has analgesic, antimalarial, antimicrobial, anti-dysentery, anti-inflammatory, and anti-tumor activities (Meena *et al.*, 2010). The young leaf shoots of *V. pubescens* are consumed raw for the treatment of hypertension, however; the roots were taken as tea to alleviate backache, and fatigue. This plant is also used produce charcoal (Oramahi and Yoshimura, 2013). *V. pubescens* can act as an antifungal, anti-termite agents and possesses insecticide activity (Meena *et al.*, 2010; Meena *et al.*, 2011; Oramahi and Yoshimura, 2013). Many vitex species have been used for bug repellent and used for traditional medicines, especially pain relief of menstruation, increasing appetite, anticancer, rhinitis and stamina, depression, venereal diseases, malaria, asthma, allergy, skin diseases, snake bite, inflammation and body pains (Lenny *et al.*, 2015; Povi *et al.*, 2015).

*V. pubescens* has been reported to produce many chemical compounds from it bark such andrographolide, methyl p-hydroxybenzoate, pinnatasterone, turkesterone, luteolin, iso-orientin and vetixin (Meena *et al.*, 2010; Lenny *et al.*, 2015). Pinnatasterone exhibited low biological activities in the pupariation tests with housefly larva. In addition, its flower have some isolated compounds for instance; flavonoids, luteolin, iso-orientin, and vitexin (Meena *et al.*, 2010; Lenny *et al.*, 2015). Cicerfuran which is a stilbene derivative compound has been isolated and identified in the ethyl acetate fraction for root extracts of *V. pubescens* in a previous study of Jayuska *et al.* (2012). Moreover, coumarin was identified from chloroform extract of the leaf that has
a higher cytotoxicity on myeloma cell (12, 73 µg/mL) in vitro (Rakhmawati & Wahyuono, 2006). Some structures of selected compounds of *V. pubescens* are illustrated in Table 2.4

**Table 2.4:** Chemical compounds from *V. pubescens* bark and leaves (Coumarin) and their biological activities.

<table>
<thead>
<tr>
<th>Name of compounds &amp; Bioactivities (V. pubescens)</th>
<th>Structure of compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Andrographolide</strong> Anti-inflammatory, anticancer, antitumor, immunology, antidiabetes antimicrobial and antivirus (Lenny, 2015)</td>
<td><img src="image" alt="Andrographolide structure" /></td>
</tr>
<tr>
<td><strong>Methyl p-hydroxybenzoate</strong> Antifungal (Lenny, 2015)</td>
<td><img src="image" alt="Methyl p-hydroxybenzoate structure" /></td>
</tr>
<tr>
<td><strong>Pinnatasterone</strong> Pupariatio (Meena, 2010)</td>
<td><img src="image" alt="Pinnatasterone structure" /></td>
</tr>
<tr>
<td><strong>Turkesterone</strong> Increase ATP synthesis (Meena, 2010)</td>
<td><img src="image" alt="Turkesterone structure" /></td>
</tr>
<tr>
<td><strong>Luteolin</strong> Antioxidant and antitumoral activity (Meena, 2011)</td>
<td><img src="image" alt="Luteolin structure" /></td>
</tr>
<tr>
<td><strong>Iso-orientin</strong> Hypoglycaemic component (Meena, 2011)</td>
<td><img src="image" alt="Iso-orientin structure" /></td>
</tr>
<tr>
<td><strong>Vitexin</strong></td>
<td>Inhibits thyroid peroxidase (Meena, 2011)</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td><img src="image1" alt="Vitexin molecule" /></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Coumarin</strong></th>
<th>Antioxidant and anticancer (Rakhmawati &amp; Wahyuono, 2006)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image2" alt="Coumarin molecule" /></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Medicinal plants

*C. barometz* and *V. pubescens* leaves were identified and collected freshly from the Herbarium of Rimba Ilmu, University of Malaya (UM), Kuala Lumpur with voucher numbers KLU 48648 and KLU 48647, respectively. The two plants authenticated by Dr. Sugumaran Manickam, a botanist at Institute of Biological Sciences (IBS), Rimba Ilmu Botanic Garden, University of Malaya.

3.1.2 Main chemicals

*(1) Omeprazole*

Omeprazole is the reference antiulcer drug used in the gastric ulcer experiments in this study to evaluate the gastro-protective activity of ethanol extracts of each plant in animals. It belongs to the proton pump inhibitors (PPIs) family that has been used for treatment of gastric and peptic ulcers by reducing acidity. This reference drug was purchased from the University Malaya Medical Centre (UMMC) Pharmacy. Omeprazole (20 mg/kg) was dissolved in 10% Tween 20 and orally administered to the SD rats at a dose of 20 mg/kg body weight (5 mL/kg) (Amaral *et al.*, 2013).

*(2) Intrasite gel*

Intrasite gel is the reference drug used in the study of wound healing experiments to evaluate the healing potential of wounds. It is manufactured for wound care use by Smith and Nephew Healthcare Limited. It was purchased from the UMMC. This gel is a transparent, sterile and non-adherent amorphous hydro-gel, which contains <10% modified carboxymethyl cellulose (CMC), 10% to 30% propylene glycol, and
> 60% water. It is having moisturizing properties and bacteriostatic effects. The main actions of gel were absorbing exudates and toxins from the wound surface, preventing slough formation, rehydrating necrotic tissue, and removing slough without fragile granulation tissues damage or destruction of the viable surrounding skin tissues (Hajiaghaalipour et al., 2013).

(3) **Streptozotocin**

Streptozotocin (STZ) is an antibiotic and nitrosourea analogue that creates pancreatic islet \( \beta \)-cell destruction and is widely used experimentally for induction non-insulin-dependent diabetes mellitus (Type II) NIDDM. It inhibits the secretion of insulin and causes an insulin-dependent diabetes mellitus. STZ (Sigma - Aldrich, Germany) was used in this research before processed to wound healing activity study in diabetic rats as described previously by Kumar et al., (2012).

In this study, NIDDM was induced experimentally after overnight fasting of healthy adult *Sprague Dawley* (SD) male rats (208-235 g) by a single intraperitoneal injection of 50 mg/kg body weight STZ dissolved in 0.5 mL/rat cold citrate buffer (pH 4.5). This solution preparation was immediately prepared before use. The glucose of blood in each serum of each rat was measured before and post STZ injection. The producing of STZ-induced insulin deficiency leads to hyperglycemia in rats. Therefore, the blood samples were taken from tail vein of rats to confirm and measure the elevated glucose levels in plasma after injection at 72 hrs and on the 7\(^{th}\) day using Accu-check glucose test (strips) and glucose meter (Accu-check, Roche Diagnostics, USA) (Simeonova et al., 2016). Only diabetic rats that had a certain glucose range (>7 mmoL and <20 mmoL) were used for the anti-diabetic study.
(4) *Ketamine and xylazine (Anaesthetic agents)*

The anaesthesia was prepared by mixing 0.01 mL xylazine (100 mg/mL, Ilium, Australia) and 0.09 mL ketamine (100 mg/mL, Ilium, Australia). Each rat was injected intraperitoneally with 0.2 mL/200 g body weight of the ketamine/xylazine mixture before sacrificing (Wasman *et al.*, 2011).

### 3.1.3 Experimental animals

Healthy adult SD rats (6–8 weeks old) within the range of the body weight of 162–190 g were used in the acute toxicity test. On the other hand, the range of body weight of healthy adult male SD rats (6-9 weeks old) in the gastroprotective experiments was 184-209 g and 208-235 g for wound healing evaluation experiments.

The animals in this work were obtained from Experimental Animal House, Faculty of Medicine, University of Malaya and the work was designed to minimize the number of required animals and their suffering. They were housed singly in cages with a wide-mesh wire bottom to prevent coprophagy and dominancy. All rats were kept at 25°C of room temperature and in humidity rooms on a standard light/dark cycle (12 hrs light/dark cycle) (Simeonova *et al.*, 2016). For two days, the animals were left to acclimatize to the animal room conditions and they were fed a standard pellet diet and tap water (OECD, 2005).

The Ethics Committee for Animal Experimentation, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia approved the cited technique in this study with the Ethic No. PM/30/05/2012/NSIAW(R). Through the experiments, all animals received suitable animal care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences (Garber *et al.*, 2010).
3.2 The experimental design of study

This study was divided into two main studies: *in vitro* and *in vivo* studies (Figure 3.1). The *in vitro* study included the assessments of antioxidant activities, cytotoxicity (MTT assay), identification of active compounds and gene expression. However, the *in vivo* study included the evaluations of acute toxicity and gastroprotective effects and wound healing potential on SD rats. The flow charts of acute toxicity test, gastoprotective and wound healing potential studies are shown in Figures 3.2, 3.3, 3.4 and 3.5.
Figure 3.1: The experimental design of the study.
Figure 3.2: Flow chart of the acute toxicity test.
**Figure 3.3:** Flow chart of the gastro protective study.
Figure 3.4: Flow chart of wound healing potential study in normal rats.
Figure 3.5: Flow chart of wound healing potential study in diabetic rats.
3.3 Medicinal plants extractions and preparations

The extractions method of medicinal plants used usually to obtain the effective extracts and extract the active compounds of the plants then use in various scientific researches either in vivo or in vitro studies for different purposes.

In the beginning of extraction, the collected plants of this study were tape washed, shade dried for 4 to 5 days and ground to powder by electric blender. 100 g of dried leaves were soaked for 4 days in 900 mL ethanol 95% in a laboratory glass bottle (1L) and stirred daily. After that, the filter paper (Whatman No. 1, England) was used to filter the extracts and the extraction distillation was dried under reduced pressure in a Buchi Rotary Evaporator R-215 (Chemopharm Sdn. Bhd., Switzerland) (Luque & Priego-Capote, 2010). The C. barometz leaves yielded crude extracts 18.1% (green) (w/w) though V. pubescens leaves yielded crude extracts 9.7% (dark-green) (w/w). Each dried EtOH (ethanol) extract was kept at -20°C in freezer (Panasonic, Japan) prior to usage.

The previous extracts were used in vivo and in vitro experiments. DMSO was used as a solvent of the plant extracts (99 % for antioxidant in vitro and 0.2 % for cytotoxicity in vitro) for the extracts preparation the in vitro experiments as antioxidant evaluations. However, in vivo experiments for acute toxicity test; each extracted plant was dissolved in 10% Tween 20 and administered orally to experimental rats at doses of 2 g/kg and 5 g/kg. On the other hand, for anti-ulcer activity against ethanol-induced gastric mucosal injury, the extract of plants was dissolved in 10% Tween 20 (w/w) at doses of 62.5, 125, 250, and 500 mg/kg body weight for oral administration, in accordance with earlier reports (AlRashdi et al., 2012). Moreover, each extract was dissolved in 2% CMC (w/v) with 100 mg/mL and 200 mg/mL doses for wound healing induction in SD rats (Mahmood et al., 2010).
3.4 *In vitro* study

3.4.1 Antioxidant activity

The medicinal plants of this study were investigated to determine their antioxidant activities. The Antioxidant assays *in vitro* are the ferric-reducing antioxidant power (FRAP), scavenging of diphenyl-picrylhydrazyl radicals (DPPH), total phenolic content (TPC), total flavonoid content (TFC), superoxide anion $O_2^-$ and nitric oxide (NO’).

3.4.1.1 Ferric-reducing antioxidant power (FRAP) assay

The reduction of Fe$^{3+}$-TPTZ to complex Fe$^{2+}$-TPTZ is the basis of the principle of FRAP assay. Fe$^{3+}$-TPTZ is colourless or light brownish is reduced to the blue colour complex Fe$^{2+}$-TPTZ at low pH if there is any antioxidant (reductant). The FRAP of each ethanolic extract of the plants was estimated according to the recommended method (Henderson *et al.*, 2015).

i. *Chemicals*

DMSO and distilled H$_2$O in this assay were used as solvents to dissolve the controls (BHT (butylated hydroxytoluene), ascorbic acid, quercetin, and gallic acid) and the tested plants. However, FeSO$_4$ was used for the standard curve in FRAP assay. The following chemicals (Sigma–Aldrich, UK) are essential in the preparation of FRAP reagent.

1. Sodium acetate trihydrate (C$_2$H$_3$NaO$_2$ . 3H$_2$O) (pH 3.6)
2. Hydrochloric acid (HCl) 40 mM
3. 2, 4, 6-Tri (2-pyridyl)- s-triazine (TPTZ) 10 mM.
4. Ferrous (Fe (II)) chloride hexahydrate (FeCl$_3$.6H$_2$O) 20 mM
5. Glacial acetic acid (C$_2$H$_4$O$_2$)
**ii. Procedure**

In the FRAP reagent preparation, the Sodium acetate trihydrate (0.0775 g) was dissolved in 25 mL distilled H₂O then mixed with 0.4 mL of glacial acetic acid. TPTZ (0.0078 g) was dissolved in 2.5 mL of distilled H₂O previously mixed with 0.1 mL of HCl (1 M). FeCl₃·6H₂O (0.027 g) was dissolved in 2.5 mL of distilled H₂O and mixed well. 25 mL of acetate buffer, 2.5 mL of TPTZ, and 2.5 mL of FeCl₃·6H₂O solutions were mixed well at the ratio of 10:1:1 and then incubated at 37°C in the water bath for 5 mins before usage. However, the preparation of standard was a dissolve of 0.0028 g of FeSO₄·7H₂O in 10 mL of distilled H₂O. After that, different dilutions were prepared for standard curve assessment and each sample reading was referenced to the standard curve of prepared FeSO₄·7H₂O. The controls (except ascorbic acid 1 mg dissolved in 1 mL distilled H₂O and diluted 10 times) and the extract of each plant were prepared by dissolving 1mg in 1mL of DMSO (99%). Lastly, 10 μL of each plant extract, standard and controls were added to 300 μL of the FRAP reagent (triplicate) in 96-wells microplate. They were left in the dark for 4 min, then the absorbance of the reduced complex was recorded spectrophotometrically at 593 nm using a power wave ×340 ELISA Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The standard curve was constructed linearly (R² = 0.998) between 100 and 1000 M FeSO₄. The results were expressed as μmol Fe (II)/g dry weight of the extract and compared with the controls.

**3.4.1.2 Scavenging of diphenyl- picrylhydrazyl radicals activity (DPPH) assay**

The DPPH free radical is a stable organic chemical compound and the scavenging of diphenyl- picrylhydrazyl radicals’ activity assay is based on measuring of the capability of each extract to donate hydrogen electrons to the stable 2, 2-diphenyl-1-pyrrilhydrazil (DPPH) free radical in the solution. Then the dark violet colour of the DPPH reagent
changes to light yellow colour as a result of the reduction of DPPH free radicals. The capability of DPPH radical scavenging of each extracted plant was assessed according to the method described by Awah et al. (2010) with minor modifications.

\textit{i. Chemicals}

1. DPPH reagent
2. DMSO
3. Ethanol 95%
4. The controls: BHT, ascorbic acid, quercetin, and gallic acid

The DPPH reagent, absolute DMSO and ethanol 95% were purchased from Sigma-Aldrich, UK and Thermo Fisher Scientific, USA. These chemicals and the tested ethanol extract of each plant used in this assay.

\textit{ii. Procedure}

The DPPH reagent solution was prepared by dissolving and stirring 1 mg of DPPH in 25 mL of absolute ethanol until totally dissolved. All controls (except ascorbic acid 1 mg dissolved in 1 mL distilled H$_2$O that used as antioxidant standard and diluted 10 times) and the extract of each plant (1 mg/1 mL) were dissolved in DMSO (99%). The stock solution of the plant extracts, controls and standard were prepared and diluted to produce five different concentrations (50, 25, 12.5, 6.25, 3.125, 1.56 μg/mL). 5 μL of each plant extract, controls and standard were mixed separately with 195 μL DPPH (40× dilution) in 96-wells microplate (triplicate). After that, each mixture was incubated at 25°C. The absorbance value was measured for 2 hrs at 20 min intervals using a spectrophotometer of power wave ×340 ELISA Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) at 515 nm. The DPPH radical scavenging activity was calculated according to the following equation:
% DPPH radical scavenging inhibition = \{(AB – AA)/AB\} \times 100

AB is the absorption of the blank sample; AA is the absorption of the tested samples.
The inhibitory concentration 50% (IC₅₀) of each sample required to scavenge 50% of the DPPH radicals values for each sample was determined as well as the kinetics of DPPH scavenging reaction in this assay (Al-Henhena et al., 2014).

3.4.1.3 Total phenolic content (TPC) assay

Antioxidant compounds have usually phenolic form (contains OH groups) that showed antioxidant properties and destroy radicals. The theoretical method of TPC test has been proposed to estimate antioxidant activities of phenolic substances in the plants by the determination the quantity of the phenolic compounds. This assay based on the reaction of oxidation-reduction between Folin-Ciocalteu reagent solution (phosphomolybdic and phosphotungstic acid) and the phenolic compounds in the sample. The Folin-Ciocalteu reagent oxidizes phenolic groups in the sample and reduces the formation of acid (a blue colour). Moreover, the alkaline medium reaction of sodium carbonate leads to develop this blue colour (Reynaud et al., 2010).

i. Chemicals

1. Folin-Ciocalteu reagent 10% (v/v) (Merck, Darmstadt, Germany)
2. Sodium carbonate solution 10% (w/v)
3. DMSO
4. The controls: BHT, ascorbic acid, quercetin, and gallic acid

The chemicals were used in the test with the tested ethanol extract of each plant. In addition, the gallic acid is used also as a standard phenolic compound in this method.
ii. Procedure

Total phenolic content of the ethanol extract for *C. barometz* and *V. pubescens* leaves was measured using Folin-Ciocalteu method according to (Qader *et al.*, 2011). The controls, extracts and standard were prepared as previous methods (1mg/mL). Each 50 μL of sample (plant extract, controls or standard) was added in triplicate to 50 μL of the Folin-Ciocalteu reagent and incubated for 3 min in 96-wells plate. Then 100 μL of sodium carbonate solution was added and then allowed to stand for 1 hr in the dark. The absorbance was measured at 750 nm using a spectrophotometer of power wave x340 ELISA Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Then the concentration of phenolic compounds was read from the calibration line and the phenolic content in each sample was expressed as gallic acid (mg/mL) equivalent (R2 = 0.997).

3.4.1.4 Total flavonoid content (TFC) assay

Flavonoid is the product of glycoside process and has polyphenol structure compound that is the most abundant structures in the plants and soluble in the water. The TFC assay depends on an aluminium chloride (ALCl₃) colorimetric method. This assay was performed by the reaction between aluminium chloride and the C4 keto-group and either C3 or C5 hydroxyl group of the flavonoid forms acid stable complex (Chavan *et al.*, 2013).

i. Chemicals

1. ALCl₃ 10%
2. Potassium acetate (KCH₃COO) 1 M
3. Phosphate-buffered saline (PBS) (pH 7.3)
4. Quercetin (a standard flavonoid compound)
5. Absolute methanol

6. DMSO

All chemicals were purchased from Merck, Darmstadt, Germany and Sigma-Aldrich, UK.

ii. Procedure

In brief of colorimetric AlCl$_3$ method (Chavan et al., 2013), 10 μL of each plant extract (prepared in DMSO (1mg/1mL)) was separately mixed with 60 μL of methanol, 10μl of AlCl$_3$, 10 μL of 1 M potassium acetate, and 120 μL of distilled water (in triplicate) in 96-well microplate and left at room temperature (25 ºC) for 30 min. Each absorbance of the reaction mixture was measured at 412 nm using a spectrophotometer of power wave ×340 ELISA Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Then the concentration of flavonoid compounds was read from the calibration line and the total flavonoid content was expressed as quercetin (mg/mL) equivalent (R2 = 0.997).

3.4.1.5 Nitric Oxide (NO$^-$) radical scavenging activity assay

NO$^-$ is very unstable radical under aerobic conditions. The excess generation of NO$^-$ was considered as an indicator of many pathological conditions in the human body. This assay used to determine the ability of ethanol extract of each plant to counteract the nitric oxide formed from Sodium nitroprusside (SNP) (Nandhakumar & Indumathi, 2013).

i. Chemicals

1. SNP 5 mM

2. Sulfanilamide (SA) 1%
3. N-(1-naphthyl) ethylene-diamine-dihydrochloride (NED) 0.1%

4. Phosphate-buffered saline (PBS) (pH 7.3)

5. Phosphoric acid (H₃PO₄) 5%

6. DMSO

7. The controls: BHT, ascorbic acid, quercetin, and gallic acid

The previous chemicals were purchased from Merck, Darmstadt, Germany, Sigma-Aldrich, UK.

**ii. Procedure**

The NO• generated from SNP was measured according to the method of (Lalezari *et al.*, 2011). Briefly, the reaction of mixture 50 μL containing SNP in PBS, with 50 μL of each sample (The controls and extracts were prepared as previous methods (1mg/mL) in different concentrations) started after mixing together in 96-wells microplate (in triplicate). After that, they were incubated at 25°C for 1hr in front of a visible polychromatic light source (25 W tungsten lamp). The generated NO Radical interacted with oxygen to produce the nitrite ion (NO₂⁻) which was measured after adding 100 μL of an equal amount of Griess reagent (SA in H₃PO₄ and NED) on the incubation mixture. The absorbance of the formed chromophore (purple azo dye) was analysed at 550 nm using a spectrophotometer of power wave ×340 ELISA Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The generated NO₂⁻ of each plant was estimated using a standard curve based on nitrate (NO₃⁻) solutions of known concentrations (R² = 0.998). The percentage of scavenging effect was calculated using the same calculation way of the O₂•⁻ radical scavenging assay.
3.4.1.6 Superoxide anion (O$_2^-$) radical scavenging activity assay

O$_2^-$ radical scavenging assay was based on the capacity of the plant to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). O$_2^-$ radical affects many cellular functions such as damaging oxidizing proteins and reaction-center pigment (Thatoi et al., 2014).

i. Chemicals

1. B-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH) 468 μM.

2. Nitroblue tetrazolium (NBT) 150 μM

3. Phenazine methosulfate (PMS) 60 μM

4. Phosphate buffer saline (PBS) (pH 7.3)

5. Phosphoric acid (H$_3$PO$_4$)

6. The controls: BHT, ascorbic acid, quercetin, and gallic acid

ii. Procedure

The superoxide anion scavenging activity of ethanolic extract of plant was performed according to the method of Awah et al. (2010) with some modifications. Superoxide radicals were produced in PMS-NADH systems by NADH oxidation and assayed by NBT reduction. In this assay, the superoxide radicals were generated in 150 μL of PBS in triplicate containing 50 μL of NBT solution, 50 μL NADH solution and 50 μL of different concentrations of each sample (The controls, extracts and standard were prepared as previous methods (1mg/mL)) in 96-well (in triplicate). The reactions were started by adding 50 μL of PMS solution to the mixture (Chanda & Baravalia, 2010).
After that, it was incubated at room temperature for 10-15 min in dark and the absorbance was measured at 570 nm against blank sample using a spectrophotometer of power wave x340 ELISA Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). (R2 = 0.958). The percentage of scavenging effect was calculated using the following equation:

\[
\text{\% } \text{O}_2^- \text{ radical scavenging inhibition} = \left\{ \left( \frac{\text{AB} - \text{AA}}{\text{AB}} \right) \times 100 \right\}
\]

i. AB is the absorption of the blank sample; AA is the absorption of the tested samples.

The IC\(_{50}\) of each ethanol extract of plant was calculated graphically using a calibration curve in the linear range by plotting the samples concentration.

### 3.4.2 Fractionation and identification of the active compounds

#### 3.4.2.1 Fractionation of the crude extracts

i. **Chemicals**

1. Distilled H\(_2\)O
2. Methanol

The absolute methanol was purchased from Merck, Darmstadt, Germany. Distilled H\(_2\)O and absolute methanol were used as solvents in this method.

*C. barometz* and *V. pubescens* leaves crude extracts were fractionated separately using reversed phased column, Luna 5u C18(2) 100A (phenomenex), dimensions (150 x 21.20 mm and particle size, 5.0 μm) with preparative High Performance Liquid Chromatography (HPLC) (PREPLC purificat and multiple wavelength detectors (Kromaton, Interchim), puriflash 4250-250, UK). The mobile system that used was 90:10 (water: methanol) to 100% (methanol) in 180 min. The
HPLC parameters were 6 mL of volume of injection and 5 mL/min of flow rate and the HPLC gradient conditions listed in Table 3.1.

After that, the fractions of each plant were obtained and collected. The crudes and the collected fractions used for cell viability assessment in vitro using MTT assay with WRL-68 cell line (Taha et al., 2012). Then the active fractions (Fraction 1 of *C. barometz* and Fraction 2 of *V. pubescens*) continued separately with a liquid chromatography mass spectrometry (LC-MS) analysis to identify the mass of active compounds (Di et al., 2013).

<table>
<thead>
<tr>
<th>Table 3.1: HPLC gradient conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>180</td>
</tr>
</tbody>
</table>

3.4.2.2 Identification of active compounds by LC-MS

i. Chemicals

1. Distilled H₂O
2. Acetonitrile

The absolute methanol was purchased from Merck, Darmstadt, Germany. Distilled H₂O and acetonitrile were used as solvents in this method.

Agilent technologies 6490 series triple quad LC-MS (QQQ) mass spectrometer with dual ESI source, G6490, Singapore was used to identify the active compounds of the active fractions of *C. barometz* (*Cb*) leaves (F1) and *V. pubescens* (*Vp*) leaves (F2). The LC-MS gradient conditions listed in Table 3.2 using 90:10 (water: acetonitrile) to 100% (acetonitrile) in 10 min. The LC-MS parameters were 1µL of volume Injection, 0.5
mL/min of flow rate and the XBridge C18 2.5 μm 2.5 x 50 mm columns (Waters, Ireland) was used in this procedure.

Table 3.2: LC-MS gradient conditions

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Distilled H₂O %</th>
<th>Acetonitrile %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Lastly, the identified compounds of CbF1 (Cibotium barometz Fraction 1) and identified compounds of VpF2 (Vitex pubescens Fraction 2) were investigated based on mass of charge (m/z) (Masiá et al., 2013). The data of LC-MS was processed and analysed using the agilent mass hunter qualitative analysis B.06.00.

3.4.3 MTT assay and cytotoxicity in the cell line

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide MTT assay was performed to examine the *in vitro* cytotoxic properties of the *C. barometz* and *V. pubescens* leaves crude extracts and their fractions on hepatic human cell line WRL-68 and Fibroblast cells (Taha et al., 2012). The cell viability/proliferation (the number of viable cells) was measured using a standard colorimetric MTT reduction assay. The assay measures the amount of purple MTT formazan salt produced from metabolically cleaved yellow MTT tetrazolium salt. It has been established previously that the amount of MTT formazan salt produced is proportional to the amount of viable cells (Taha et al., 2012).

i. Chemicals

1. MTT formazan salt
2. DMSO (0.2%)
3. Roswell Park Memorial Institute (RPMI 1640)

4. Fetal bovine serum (FBS) 10%

5. Penicillin-Streptomycin 1%

6. WRL-68 and Fibroblast cell lines

DMSO were purchased from Sigma-Aldrich, UK whereas MTT, RPMI, FBS, Streptomyacin and penicillin were obtained from Invitrogen, Carlsbad, CA. Fibroblast and WRL-68 cells in this assay were obtained from the American Type Culture Collection (ATCC® CL48™; ATCC® CRL-1634™ respectively, Manassas, USA).

The two cell lines were cultured in RPMI 1640 growth medium that was supplemented with FBS, 100 mg/ mL streptomycin and 100 U/mL penicillin and incubated in 5 % CO₂ incubator (NuAire Inc., Plymouth, USA) at 37° C in a humidified atmosphere (Gauley & Pisetsky, 2010). The cells were harvested via detaching from the culture flask by adding trypsin when the flask became confluency. Then the assay was adapted into 96-wells microplate. Approximately, 5000 cells per well were seeded in the growth media. After 24 hrs, cells were treated for 48 hrs in triplicate for each sample. The samples included different concentrations of each plant extract and its fractions at 200, 100, 50, 25, 12.5, 6.25, 3.125 µg/mL that dissolved in 0.2% DMSO (vehicle control). Next, 10 µl MTT (5 g/mL PBS) was added to the treated cells for 4 hrs at 37°C. After the incubation, the supernatants (The medium with MTT) were carefully aspirated and 100 mL of DMSO was added to dissolve the formazan crystals product. The optical density was measured spectrophotometrically at 570 nm using a multi-wells plate reader (Asys UVM 340, Eugendorf, Austria). The IC50 (the concentration of the sample at which 50 % cell growth is inhibited) was calculated using the curve fitting of the cell viability data and the cell viability percentage was calculated according to Ng et al., (2011) from the following equation:
Cell viability % = (Abs$_{570}$ treated/ Abs$_{570}$ untreated) ×100.

3.4.4 Gene expression study

The gene expression was estimated using real time-polymerase chain reaction (RT-PCR) to evaluate the molecular response of the fibroblast cells toward the treatments of crude extract of C. barometz leaves (the best effective plant, in vitro study). The human skin fibroblast cell line (Hs27) fibroblast cells were used to evaluate the expression of Bax (Hs00180269_m1), Collagen Type I Alpha 2 (Col1a2) (Hs00164099_m1) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (Hs03929097_g1). The endogenous control housekeeping gene Gapdh was used for the normalization.

3.4.4.1 RNA Extraction

RNeasy® plus mini kit (Cat. No. 74134, QIAGEN, Germany) was used to isolate ribonucleic acid (RNA) from the samples. The kit contains Buffer RLT, Buffer RW1, Buffer RPE, RNase-Free Water, RNeasy Mini Spin Columns, Collection Tubes (1.5 mL), Collection Tubes (2 mL), RNase mini spin column and gDNA eliminator mini spin column.

The RNA was extracted from the Hs27 fibroblast cells (ATCC® CRL-1634™) cultured in 25 cm flask according to the manufacture protocol in the following steps:

1. The fibroblast cells were seeded in 25 cm flasks and were treated with the C. barometz leaves ethanol extract (200 mg/mL). The untreated cells were used as a control.
2. The grown cells in cell-culture flasks were trypsinized and collected as a cell pellet prior to lysis.
3. The cells were disrupted by adding Buffer RLT then homogenized by pipetting the lysate directly into a QIA shredder spin column.
4. They were placed in 2 mL collection tubes and centrifuged for 2 min at full speed.

5. The homogenized lysate was transferred to gDNA Eliminator spin column placed in 2 mL collection tubes and centrifuged for 30 sec at 10000 rpm.

6. The columns were discarded and 350 μL of 70% ethanol was added to the flow-through.

7. They were mixed well by pipetting and transferred 700 μL of the RNeasy spin column placed in a 2 mL collection tube and centrifuged for 15 sec at 10000 rpm. The flow-through was discarded.

8. To eliminate genomic DNA contamination, 10 μL DNase I stock solution and 70 μl buffer RDD were added directly to the RNeasy spin column membrane to remove DNA (QIAGEN, Hilden, Germany, Cat.No:79254).

9. The previous mixture was incubated for 20 min at room temperature (25°C).

10. The membrane was washed twice with 500 μL of RPE washing buffer and centrifuged for 15 sec at 10000 rpm.

11. RNA was collected by placing RNeasy spin column in a new 1.5 mL collection tube and adding 30–50 μL RNase-free water. The purified RNA was kept at -80°C until used.

12. The RNA purity and integrity: The pure isolated RNA may be contaminated by DNA, protein or phenol in the RNA extraction. Therefore, evaluating of RNA integrity and purity is necessary. The purity and concentration of RNA were assessed by A260/280, A260/230 UV absorption ratios (Nano-drop 2000 Spectrophotometer, Thermo Scientific Waltham, Massachusetts, USA). Agarose gel is the most common way to evaluate RNA integrity. Each RNA sample was assessed by integrity of Ethidium- Bromide agarose gel (1 %) and evaluated by
electrophoresis in Tris/Borate/EDTA (Ethylene diamine tetra acetic acid) (TBE) buffer. Briefly, 0.5 g of agarose was added to 50 mL TBE mixed with 0.5 µL Ethidium- Bromide and heated in microwave for one minute. 5 µL of samples was added to 2 µL loading dye and incubated for 5 min at 85 °C then the agarose gel was run for 45 min in 90 V. The run of the agarose was observed in the specific gel documentation system (Vilber Lourmat, Fisher Scientific). RNA sharp and clear bands can be seen in 18S and 28S (small subunits) RNA. To catch the RNA bands location, 1Kb ladder was used.

3.4.4.2 RNA to cDNA conversion

RNA samples should be converted to complementary DNA (cDNA) to perform the two-step real time PCR method. Reverse transcription into cDNA was done using Two-Step qRT-PCR kit, High capacity RNA to cDNA by Applied Biosystems, USA (product PN4387406). It includes the reagents for reverse transcription (RT) buffer and enzyme to convert total RNA to single-stranded cDNA.

According to the manufacturer’s guidelines total RNA was converted to cDNA by adding of up to 9 µL RNA (1000 ng), 10 µL of RT buffer, and 1 µL of RT enzyme. To perform reverse transcription of converting, optimized program the thermal cycler conditions was used (MJ Research PTC-100 Thermal cycler, USA). The program was included 37 °C for 60 min, to stop reaction at 95 °C for 5 minutes and hold in 4 °C. Then the cDNA is stored at (-15 °C to -25 °C) until used in real Time-PCR.

3.4.4.3 Real time PCR running

TaqMan® Gene Expression Master Mix (No. 4444602) assay was performed according to the reaction setup instructions generated by the StepOne software (Ver. 2.0, Applied Biosystems). The expressions of selected genes were investigated in this experiment.
According to the manufactured protocol, 50 ng of cDNA in 1 μl, 5 μL of master mix buffer, 0.5 μL of each target primer, and 3.5 μL RNase free water was loaded in 96 wells plates. The total volume of PCR mixture was 10 μL in each well. All experimental samples were loaded in three biological replicates. After that, the plate was loaded into the Applied Biosystem Step One Plus thermal cycler. It was subjected to PCR amplification under the subsequent thermal cycling conditions. The conditions are preheating at 50 °C for 2 min and at 95 °C for 20 sec, followed by 40 cycles of shuttle heating at 95 °C for 1 sec (denaturation) and at 60 °C for 20 sec (annealing/extension). A negative control without cDNA (no template control) was included in this assay.

The comparative cycle threshold (C\text{T}) method quantification was estimated using delta delta cycle threshold (\Delta\DeltaC\text{T}) expression of the investigated genes that was normalized by the endogenous control housekeeping gene (\text{Gapdh}). The C\text{T} values were obtained depending on the average of triplicate measurements. C\text{T} is described as the PCR cycle number at which the fluorescence produced from amplification of the target gene within sample proliferations to a threshold value of 10 times the standard deviation of the base line emission. It is inversely proportionate to the initial amount of the target cDNA (Kheirelseid et al., 2010).

Data analysis was performed using GenEx Standard software (MultiD analyses AB, Sweden), however, for statistical analysis of real time PCR experiments, the results of a given gene were expressed as the difference between the \DeltaC\text{T} value obtained from treated versus untreated (Calibrator). The calculation of fold changes was done as designated in the following equations:

\[ C\text{T Target} - C\text{T Endogenous control} = \DeltaC\text{T} \]
\[ \Delta C_T \text{ Sample (treated)} - \Delta C_T \text{ Calibrator (untreated)} = \Delta \Delta C_T \]

Fold change = \(2^{\Delta \Delta C_T}\)

3.5 *In vivo* study

3.5.1 Acute toxicity test

The acute toxicity study was performed to determine a safe dose of each plant according to Organization for Economic Cooperation and Development (OECD) guidelines (OECD, 2005). This test is used to describe the adverse effects of the substance that may result from single or multiple exposures within a 24 hrs period; therefore, the test was used for determination a safe dose of each plant in this study.

3.5.1.1 Chemicals

1. Tween 20, 10%
2. Buffered formalin 10%
3. Haematoxylin and Eosin (H & E) staining
4. Ethanolic extracts of *C. barometz* and *V. pubescens*

Tween 20 and buffered formalin were purchased from Merck, Darmstadt, Germany but H & E staining was purchased from Sigma, USA. Tween 20 used in the animal experiment as vehicle and solvent of each plant in the test and prepared as explained in the following section 3.5.1.3.

3.5.1.2 Procedure of animal experiment

For each plant of this study, thirty-six SD rats (18 males and 18 females) were randomly divided equally into 3 groups labelled as shown in the figure 3.2. This study included the groups of vehicle (10% Tween 20, 5 mL/kg), low dose 2 g/kg, and high dose 5 g/kg of each ethanolic plant extract (Hor *et al.*, 2011). Prior to dosing, the
animals were fasted overnight (food but not water) then food was withdrawn for a further 3 hrs after dosing. The animals were observed for 30 min and 2, 4, 8, 24 and 48 hrs after administration for the commencement of toxicological symptoms. Mortality was recorded over a period of two weeks.

On the 15th day, the animals were injected with an over-dose of anaesthesia (xylazine with ketamine) as explained in section 3.1.2. They were sacrificed to collect blood in gel-activated clot tube to centrifuge at 3000 rpm for 10 min then 1 mL of serum was transferred to a clean micro-centrifuge tube for biochemical analysis (Adjei et al., 2014). The blood-serum of all collected samples were analysed in the Clinical Diagnostic Laboratory of the University Malaya Medical Centre (UMMC). The liver, kidney function tests and lipid profile tests were the main biochemical tests of toxicity evaluation in the blood. The analysed biochemistry parameters of serum were total protein, albumin, globulin, total bilirubin, conjugated bilirubin, ALP, AST, ALT, GGT for liver function tests, serum electrolytes (sodium, potassium, chloride and CO₂), anion gap, urea and creatinine for kidney function tests and triglyceride, total cholesterol and HDL cholesterol for lipid profile tests (Chung et al., 2012). On the other hand, the collected liver and kidney of each rat were cut and fixed in 10% buffered formalin for 24 hrs for histological staining examination (Halabi et al., 2014).

3.5.1.3 Procedure of haematoxylin and eosin (H&E) staining

The liver and kidney tissues were passed through several steps before staining which were fixations, tissue processing and embedding steps.

i. Fixation

Tissue sample was fixed in 10% buffered formalin for 24 hrs. It was prepared by mixing 10 mL of formaldehyde in 90 mL of PBS. The buffered formalin is a good
fixative solution of tissue. It affects cross-link membrane proteins through forming covalent bonds to preserve volume and shape of tissue from degradation, maintain cell structure. The fixation process is important to prevent autolysis and bacterial attack, preserve the volume and shape of tissue, prepare the tissue sections for clean staining and leave the tissue as close to its living state as possible.

**ii. Tissue processing**

Tissue processing is useful for removal the water and the aqueous fixative from tissues that is replaced by a medium facilitates the cutting of tissue sections. It was conducted in an automatic processor (Leica TP 1020, Germany) and performed as the following steps:

1. Dehydration: Each tissue cassette was transferred by series dilutions of ethanol starting with 50% and ending with absolute alcohol.
2. Clearing: Each tissue cassette was put twice in xylene to remove the alcohol.
3. Impregnation: Every tissue was put in a wax bath containing molten paraffin wax for 6 hrs to 8 hrs. The wax penetrated the tissues and increased the hardness of the tissues to get rid of aqueous fixative and tissue water

**iii. Embedding**

In this step, the tissues were put in molds together with liquid paraffin wax using the embedding machine (Leica Histo-Embedder, Germany) for supporting the tissue during microtomy. The embedded tissue was left to harden then stored in a refrigerator until used in sectioning.
iv. Sectioning

The microtome with a sharp blade (Leica, Germany) was used to cut the tissue into 5 μm-thick sections. The sections of tissues were gently placed in the water bath (45 °C) to remove wrinkles and picked up with clean microscopic slides for staining H & E and Masson’s trichrome stains, however; poly-L-lysine coated slides used for immunohistochemically stains. After that, all slides stored in an oven at 60 °C for 24 hrs to increase the adherence of tissue and sort out any creases obtained during sectioning or floating before staining (Halabi et al., 2014).

v. Staining

Commonly H & E staining is used in histopathology and histology. Haematoxylin is a permanent stain of the nuclear network in the tissue with blue colour though eosin is an acidic dye used as a counter stain to create red colour of cytoplasm. The steps of staining were mentioned in the appendix A.

3.5.2 Gastro protective effect study

3.5.2.1 Gastro protective experiment

i. Chemicals

1. Tween 20, 10%
2. Absolute ethanol
3. PBS
4. Sodium hydroxide (NaOH) 0.1 N
5. Buffered formalin 10%
6. Ethanolic extracts of C. barometz and V. pubescens
Tween 20, PBS, NaOH, ethanol and buffered formalin were purchased from Merck, Darmstadt, Germany. 10% Tween 20 used in this experiment as vehicle and solvent of each plant in the test (Saleh et al., 2016) and prepared as mentioned previously in section 3.3.

**ii. Ethanol-induced gastric ulceration**

Healthy adult male SD rats were distributed into seven groups (6 rats each group) for each plant. Before the experiment, they were food fasted for 24 hrs and water fasted for 2 hrs. The rats were housed in wire-bottomed cages to prevent coprophagy.

Group 1 (vehicle group) and Group 2 (ulcer control group) were administered orally with 10% Tween 20 (5 mL/kg).

Group 3 was given 20 mg/kg omeprazole orally, as the reference control group.

Groups 4, 5, 6 and 7 were given orally dosages of 62.5, 125, 250, and 500 mg/kg of ethanol extract of plant, respectively. One hour later, Group 1 was administrated with 10% Tween 20 (5 mL/kg) and Groups 2-7 were given absolute ethanol (5 mL/kg) (Rahim et al., 2014). After 1 hr, all rats were anesthetized intraperitoneally using overdose of anaesthesia (xylazine with ketamine) as explained in section 3.1.2, followed by cervical dislocation. All stomachs of rats were tied at the pyloric and cardiac ends, excised quickly, and kept in PBS. The stomachs were analyzed to assess the gastric mucus content, acidity, gross gastric lesion, biochemical parameters such as Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and malondialdehyde (MDA) and histological evaluation (Halabi et al., 2014).
3.5.2.2 Measurement of mucus content and gastric juice acidity

Each stomach was opened along the greater curvature. For each rat, the gastric juice was collected in the test tube then centrifuged at 4000 rpm for 10 min. The precipitation of centrifuged gastric juice, the mucus content, was balanced using a precision electronic balance to get the mucus weight. However, the supernatant was analysed for hydrogen ion concentration using pH meter titration with 0.1 N NaOH (Ibrahim et al., 2012).

3.5.2.3 Gross gastric lesion evaluation

The gastric mucosa of each rat was examined for injuries after gastric juice collection. The ulcers of gastric mucosa appeared as extended bands of haemorrhagic lesions parallel to the long axis of the gut; therefore, the gross gastric lesion was evaluated in this study. The length and width of the ulcer (mm) was measured with a plan meter (10 × 10 mm² = ulcer area) under a dissecting microscope of a square-grid eyepiece (1.8x). The ulcer area was determined by counting the number of small squares, 2 mm × 2 mm = 4 mm², covering the length and width of each ulcer band. According to the recommendation of AlRashdi et al., (2012), the calculation of all lesions areas in each stomach was applied by the calculation of ulcer area (UA) where the sum of small squares × 4 × 1.8 = UA (mm²). Then the inhibition % of ulcer was calculated using the following formula.

\[
\text{Ulcer inhibition} \% = \left\{ \frac{\text{UA control} - \text{UA treated}}{\text{UA control}} \right\} \times 100
\]

3.5.2.4 Histological gastric lesions evaluation

i. H & E staining

The gastric wall specimens from each rat were fixed in 10% buffered formalin, processed and embedded in the paraffin tissue-processing machine (Leica, Germany).
Sections of the stomach were prepared at a thickness of 5 μm and stained with haematoxylin and eosin (H & E) for histological and tissue architecture estimation as described previously in section 3.5.1.3 (Li et al., 2013).

**ii. Mucosal glycoprotein (PAS) staining**

Periodic acid–Schiff (PAS) staining was used to evaluate the neutral mucus production in the gastric tissue and assess the variations in glycoproteins (acidic and basic). The process of staining is a histochemical reaction that the periodic acid oxidizes neutral substances to aldehydes and reacts with the Schiff’s reagent to produce magenta colour.

Selected slides of tissue sections were stained using PAS following the manufacturer’s instructions (Sigma Periodic Acid- Schiff Commercial Kit, USA).

Each tissue section was deparaffinised and hydrated in water like the first step of H & E staining. After that, each slide was put in 0.5% periodic acid for 10 min then washed with tap water. Next, the slides were put in Schiff’s reagent for 20 - 30 min then washed with running water for 5 min. The tissue sections were counterstained with haematoxylin for 2 - 3 min. Then the slides were washed with running tap water, dehydrated with alcohol, cleared with xylene, and finally mounted similar to the H & E staining method (Sidahmed et al., 2013).

**iii. Immunohistochemistry staining**

Immunohistochemistry staining was performed to detect tissue antibodies. ARKTM (Animal Research Kit) was used to detect the immunohistochemically localization of Heat Shock protein 70 (HSP70) (1:100) and Bcl-2-associated X protein
(Bax) (1:200) proteins on the study slides (Golbabapour et al., 2013). The two proteins were purchased from Santa Cruz Biotechnology, Inc., California; USA.

Briefly, each tissue section was heated at 60°C for approximately 25 min in a hot air oven (Venticell, MMM, Einrichtungen, Germany). The tissue section of slide was de-paraffinized in xylene and rehydrated using graded alcohol. Antigens were retrieved via microwave boiling of the sample in 10 mM sodium citrate buffer. Endogenous peroxidase was blocked using 0.03% hydrogen peroxide containing sodium azide. The tissue section was washed gently with washing buffer, then incubated with HSP70 (1:500) or Bax (1:200) biotinylated primary antibodies for 15 min. After that, the sections were washed gently with wash buffer and put in the buffer bath. Each slide was kept in a humidified chamber. Then the sections were incubated for 15 min with streptavidin–HRP (streptavidin conjugated to horseradish peroxidase in PBS containing an anti-microbial agent). After that, the tissue sections were soaked gently in the washing buffer and put in the buffer bath before being incubated with diaminobenzidine -substrate - chromagen for 5 min. Then the sections were counterstained and washed with hematoxylin for 5 sec, dipped in weak ammonia (0.037 M/L) 10 times, and finally rinsed with distilled H₂O prior to the mounting of cover slips. Positive outcomes of immunohistochemical staining were perceived as brown coloration in the tissue sections under a light microscope (Halabi et al., 2014).

3.5.2.5 Antioxidant activity of tissue homogenate

The tissue homogenate of each tissue was collected and prepared for measurement the antioxidant activities and lipid peroxidation levels.
i. Preparation of tissue homogenate

The collected samples of each gastric tissue were washed thoroughly with ice-cold PBS. About 0.5 g of whole gastric tissue was weighed to prepare the tissue homogenates (10% (w/v)) and each sample was homogenized on ice with 5 mL of 50 mM PBS (pH 7.4) using a Teflon homogenizer (Polytron, Heidolph RZR 1, Germany). Then each homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C by refrigerated centrifuge Rotofix 32 (Hettich Zentrifugen, Germany). The supernatant was used for investigation of antioxidant activities, in vivo. The protein concentrations (mg/mL tissue) were estimated with Bradford’s solutions (Amresco LCC. Co., USA). 100 μL of the Bradford’s solution was added to 10 μL of each homogenate (triplicate in 96-wells microplate) with several concentrations. After the incubation of plates for 2 min, the absorbance was recorded at 595 nm (Garcia et al., 2014).

ii. Measurements of antioxidant activities of tissue homogenates

Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities of the gastric tissues were measured using commercial kits (NO. 706002, 707002 and 703102, respectively, Cayman Chemical Co., Ann Arbor, USA). The manufacturer protocols were used for the determination of the activities in the tissue supernatant of each sample.

A. SOD activity measurements

In this assay, SOD evaluation is based on the generation of superoxide radicals (O2⁻) created by xanthine and xanthine oxidase, which react with NBT to procedure formazan dye. The superoxide radical (O2⁻) is dismutate into O₂ and H₂O₂, which results in formazan formation in the presence of SOD.
Firstly, 20 μL of SOD standard was diluted in 1.98 μL of the diluted sample buffer to obtain the SOD stock solution. After that, specific volumes of the stock solution (0, 20, 40, 80, 120, 160, 200 μL) were added into 7 test tubes (labelled from A to G) separately and respectively with the corresponding volumes of sample buffer (1000, 980, 960, 920, 880, 840 and 800 μL) to prepare the SOD standards. Next, the assay was performed in duplicate in 96-wells microplate as the following steps:

1. SOD standard wells: 200 μL of the diluted radical detector was mixed to each well with 10 μL of the standard SOD/well in duplicate.

2. Sample wells: 200 μL of the diluted radical detector and 10 μL of the sample (From the H well) were mixed together into each well in duplicate.

3. The 20 μL of diluted xanthine oxidase was added to each well rapidly.

4. The 96-wells microplate was shaken carefully for few seconds to mix the contents then incubated for 20 min at room temperature. Then the plate was read by a spectrophotometer at 440 nm to 460 nm.

**B. CAT activity measurements**

The measurement of CAT activity is based on the enzyme reaction with methanol in the presence of an optimal concentration of H₂O₂.

In this assay, 10 μL of CAT formaldehyde standard was diluted in 9.99 mL of the diluted sample buffer to have a formaldehyde stock solution. Then several volumes of the formaldehyde stock solutions (0, 10, 30, 60, 90, 120, 150 μL) were added into 7 test tubes (labelled from A to G) with the corresponding volumes of sample buffer (1000, 990, 970, 940, 910, 880, 850 μL) to obtain the serial dilution s of formaldehyde standard solutions. Next, the assay was performed in duplicate in 96-wells microplate as the following steps:
1. The diluted assay buffer 100 μL, 30 μL of methanol and 20 μL of formaldehyde standard were added in each well in duplicate.

2. About 100 μL of the diluted assay buffer, 30 μL of methanol, and 20 μl of the diluted CAT (positive control) were put in two wells, however; 100 μL of the diluted assay buffer, 30 μL of methanol, and 20 μL of the sample were added to two other wells.

3. By adding 20 μL of the diluted H$_2$O$_2$ to all wells, the reaction started. Then the plate was incubated on a shaker for 20 min at room temperature 25°C.

4. The diluted potassium hydroxide 30 μL was added to each well to terminate the reaction, followed by the addition 30 μL of the CAT Purpald (chromogen). The plate was incubated on a shaker for 10 min at room temperature.

5. The CAT potassium periodate 10 μL was added to each well. The plate was incubated on a shaker for 5 min at room temperature. The absorbance of each sample was read at 540 nm.

C. GPx activity measurements

GPx catalyzes the reduction of hydro peroxides (H$_2$O$_2$) by reducing glutathione to protect the cell from oxidative destruction. This test is based on the oxidation of the reduced glutathione by GPx, coupled with the oxidation of NADPH by glutathione reductase,

1. Non-enzymatic wells (blank) were prepared by adding and mixing of 120 μL of the assay buffer with 50 μL of the co-substrate mixture in 96-wells microplate.

2. Positive control wells (bovine erythrocyte GPx) were prepared by adding and mixing of 100 μL of the assay buffer with 50 μL of the co-substrate mixture and 20 μL of the diluted GPx (control).
3. Sample wells were prepared by adding and mixing of 100 μL of the assay buffer, 50 μL of the co-substrate mixture and 20 μL of the sample.

4. Cumene hydroperoxide (20 μL) was added to all wells (in triplicate), mixed and carefully shaken for a few seconds. Then the absorbance was read every minute at 340 nm for 5 min.

**D. Measurements of lipid peroxidation (MDA) level of tissue homogenate**

Lipoperoxidation in the tissue produce naturally malondialdehyde (MDA). Therefore, the measurement of MDA in this assay is used as indicator of lipid peroxidation. The measurement of thiobarbituric acid reactive substances (TBARS) is a well-established method for detecting and monitoring lipid peroxidation. MDA levels in the tissue homogenate were measured using commercial kits (NO. 10009055, Cayman Chemical Co., Ann Arbor, USA).

The MDA standard stock solution (125 μM) was prepared by dilution of 250 μL of the MDA standard in 750 μL of distilled H₂O. Specific volumes of the stock solution (0, 5, 10, 20, 40, 80, 200, 400 μL) were added into 8 test tubes (labelled from A to H) separately and respectively with the corresponding volumes of distilled H₂O (1000, 995, 990, 980, 960, 920, 800 and 600 μL) to obtain the MDA standards. Next, the assay was performed in duplicate in 96-wells microplate as the following steps:

1. Each 100 μL of sample or standard was mixed with 100 μL of the sodium dodecyl sulfate (SDS) solution in a 5 mL vial and vortexed using the vortex.

2. The colour reagent (4 mL) was added to the mixture forcefully down the side of each vial.
3. The vials were covered with cap vials, put in a holder vertically in a boiling water bath for 1 hr then all vials were transferred immediately to an ice bath and incubated on ice for 10 min then centrifuged at 4000 rpm for 10 min at 4°C.

4. About 150 μL from each vial was loaded in duplicate to the 96-wells microplate. The absorbance was read at 530 nm to 540 nm spectrophotometrically. The quantitative estimation of lipid peroxidation was performed by determining the concentration of TBARS in the tissue. The results of MDA of samples were calculated from the standard curve.

3.5.3 Wound healing potential study

Male SD (normal or diabetic) rats were used in the wound healing study for evaluation the wound closure area, the histology and the immunohistochemistry and for investigation the antioxidant activities and levels of lipid peroxidation.

3.5.3.1 Wound healing experiment

i. Chemicals

1. CMC 2% (w/v)

2. Citrate buffer (pH 4.5)

3. Ethanol 70%

4. PBS

5. Buffered formalin 10%

6. Ethanolic extracts of *C. barometz* and *V. pubescens*

CMC, citrate buffer, PBS, ethanol and buffered formalin were purchased from Merck, Darmstadt, Germany. CMC (2%) used in this experiment as vehicle and solvent of each plant and prepared as mentioned previously in section 3.3.
ii. Experimentially induced wound excisions

The animals were anesthetized using an over-dose of anaesthesia by intraperitoneal injection (i.p) with a mixture of ketamine and xylazine as discussed in section 3.1.2. The skin of dorsal neck of each rat was shaved by electrical shaver, disinfected with 70% ethanol. An area of uniform wound 2 cm in diameter (Circular area = 3.14 cm²) using circular stamp, was excised from the nape of the dorsal neck of all rats with the aid of round seal (Dhiyaldeen et al., 2014). The wounding day was considered the day 0. During the procedure, the incision of the muscle layer was avoided and the tension of skin was kept constant as the following Figure 3.6.

![Figure 3.6: The excised skin of wound (2.00 cm in diameter) in experimental SD rats at 0 day.](image)

iii. Topical Application

All groups were topically treated twice daily with 0.2 mL of the dressing substances to each corresponding treatment for 10 days.

Group 1 (vehicle) that was dressed with 2% CMC,

Group 2 (reference) that was dressed by Intrasit gel,
iv. Wound closure area evaluation

On the 5th and 10th day, the wound closure area (A) was measured by placing a transparent tracing paper over the wound and tracing it out. The tracing paper was placed on a 1 mm² grid graph sheet, and traced out. The squares were counted and the area of healed wound was recorded. The percentage of wound closure was measured, using the initial and final area drawn on transparent paper and read by graph paper during the experiment by the following formula as used in previous studies (Dhiyaaldeen et al., 2014).

\[
\% \text{ Wound closure} = \frac{A(0 \text{ day}) - A(5^{\text{th}} \text{ or } 10^{\text{th}} \text{ day})}{A(0 \text{ day})} \times 100
\]

At 10th day post-surgery, the healed wound was excised under anesthesia then put in PBS for homogenization and 10% buffered formalin for histological and immunohistochemistry evaluation.

3.5.3.2 Experimentally proceeded wounds in diabetic rats

Healthy adult SD male rats were experimentally diabetes-induced using STZ before processed to wound healing activity study in diabetic rats as the study of Kumar et al. (2012). The non-insulin dependent diabetes mellitus (NIDDM) was induced in overnight fasted adult SD male rats by a single intraperitoneal injection of 50 mg/kg body weight STZ dissolved in citrate buffer (pH 4.5). The procedure of experimental wound excisions in diabetic rats was similar to the procedure in sections 3.5.3.1 but the dressing was for two weeks and the measurement of wound closure area was on 10th day and 15th day.
3.5.3.3 Histological evaluation of wound tissue

**i. H & E staining**

The histology of wound tissues on day 10\textsuperscript{th} post-wounding of normal rats and 15\textsuperscript{th} post-wounding of diabetic rats were fixed in 10% buffered formalin, processed, embedded in the paraffin tissue-processing machine (Leica, Germany). Sections of the wound were made at a thickness of 5 μm and stained with haematoxylin and eosin for basic histological staining and the tissue architecture evaluation (Gwaram et al., 2012). All of the techniques and preparations are further explained previously in section 3.5.1.3.

**ii. Masson's trichrome staining**

Masson’s trichrome-stained method was used for collagen staining of the healed skin using the commercial kits (Sigma, USA). The stained sections were examined under a light microscope to observe the morphology of fibroblast, collagen deposition, angiogenesis and epithelisation.

1. The phosphortungstic acid solution (1 volume) was mixed with 1 volume of phosphomolybdic acid solution, to which 2 volumes of deionized water was added.

2. Each slide was deparaffinized with xylene, rehydrated using graded alcohol as mentioned in Section 3.5.1.3 then put in deionized H\textsubscript{2}O for 5 min.

3. Each slide was mordant in pre-heated Bouin’s solution at 60 °C for 15 min

4. The slides were cooled in tap water contained in acoplin jar and washed in running tap water for 5 min to remove the yellow color from the sections.
5. The slides were stained with Weigert’s iron hematoxylin solution for 5 min then washed with running tap water for 5 min and with distilled H$_2$O.

6. All slides were stained in Biebrich scarlet acid fuchsin for 5 min then rinsed with deionized H$_2$O.

7. The slides were put in working phosphotungstic/phosphomolybdic acid solution for 5 min, transferred directly into aniline blue for 5 min, and then rinsed with distilled H$_2$O.

8. They were put in 1% acetic acid solution for 2 min and rinsed with distilled water.

9. All slides were dehydrated with alcohol, cleared with xylene, and mounted similar to H & E staining.

iii. Immunohistochemistry

The immunohistochemistry of wound tissues on day 10$^{th}$ post-wounding of normal rats and 15$^{th}$ post-wounding of diabetic rats were fixed in 10% buffered formalin, processed, embedded in the paraffin tissue-processing machine (Leica, Germany). Sections of the wound were made at a thickness of 5 μm and stained with immunohistochemistry staining of Bax and HSP70 proteins (Golbabapour et al., 2013). All the techniques and preparations are further explained previously in section 3.5.2.4.

3.5.3.4 Antioxidant activity of tissue homogenate

i. Preparation of tissue homogenate

The wound tissue homogenate (collected on 10$^{th}$ day in normal rats but on 15$^{th}$ day in diabetic rats) was prepared as explained before in section 3.5.2.5.
**ii. Measurements of antioxidant activities of tissue homogenate**

SOD, CAT, GPx levels of the wound tissue were measured for evaluation of antioxidant activities of tissue homogenate. All the techniques and preparations were further explained in details previously in section 3.5.2.5.

**iii. Measurements of lipid peroxidation (MDA) level of tissue homogenate**

The procedure of MDA measurement was further described in details previously in section 3.5.2.5.

**3.6 Statistical analysis**

All values were evaluated as mean ± Standard error (SEM). The statistical significance of differences between groups was measured using SPSS statistical program software version 20 through one-way analysis of variance (ANOVA) with post hoc Tukey’s multiple comparison test (comparing the treated groups with vehicle group). A value of $p < 0.05$ was considered significant.
CHAPTER 4: RESULTS

4.1 Medicinal plants extractions

The leaves of *Cibotium barometz* and *Vitex pubescens* were extracted according to the method described in the Section 3.3. The *C. barometz* leaves yielded 18.1% (w/w) though *V. pubescens* leaves yielded 9.7% (dark-green) (w/w).

4.2 *In vitro* study

*In vitro* study of *C. barometz* and *V. pubescens* leaves ethanolic extracts includes the evaluations of antioxidant activities, MTT assay with cytotoxicity in the cell line, identification of active compounds and gene expression.

4.2.1 Antioxidant activity

4.2.1.1 Ferric-reducing antioxidant power (FRAP) assay

The total antioxidant activity of ethanol extract of the study plants was measured using the FRAP assay. Table 4.1 showed the reduction of ferric to ferrous ions that indicated a greater FRAP value for *C. barometz* leaves that was (915.7 ± 0.071 µmol Fe (II)/g) than *V. pubescens* leaves (723.0 ± 0.030 µmol Fe (II)/g). The two plants had less FRAP values than quercetin and gallic acid standards (1544.3± 0.012 µmol Fe (II)/g and 1774.3± 0.002 µmol Fe (II)/g, respectively) though they had higher FRAP values than BHT (butylated hydroxytoluene), and ascorbic acid (261.0 ± 0.009 µmol Fe (II)/g and 457.7 ± 0.005 µmol Fe (II)/g, respectively).

4.2.1.2 Scavenging of diphenyl- picrylhydrazyl radicals activity (DPPH) assay

The scavenging of the DPPH free radicals in *C. barometz* and *V. pubescens* leaves ethanol extracts was evaluated using the DPPH assay. Table 4.1 illustrated the inhibition concentration 50 (IC<sub>50</sub>) values of the DPPH free-radical scavenging activity
of *V. pubescens* leaf (38.3 ± 0.1 µg/mL) with Inhibition % (65.3%) and less IC₅₀ values of *C. barometz* leaf (30.1 ± 0.05 µg/mL) with Inhibition % (87.5%). Otherwise, they were compared to the standards BHT, ascorbic acid, quercetin and gallic acid. The inhibition of DPPH free-radical scavenging activity of standards were 51.63%, 64.11%, 87.52% and 55.47% with IC₅₀ value (9.1 ± 0.15 µg/mL), (4.9 ± 0.11 µg/mL), (1.8 ± 0.04 µg/mL) and (1.4 ± 0.13 µg/mL), respectively.

### 4.2.1.3 Total phenolic content (TPC) assay

The amount of phenolic compounds in plant ethanolic extracts was determined using TPC assay as shown in Table 4.1. TPC of *C. barometz* leaves (1578 ± 0.93 mg/g) was slightly higher than TPC of *V. pubescens* leaves (1565 ± 0.93 mg/g) with equivalent to gallic acid. And TPC of two plants were greater than TPC of BHT, ascorbic acid, quercetin and gallic acid standards (402 ± 0.25 mg/g, 1511 ± 0.90 mg/g, 693 ± 0.42 mg/g and 1114 ± 0.67 mg/g respectively).

### 4.2.1.4 Total flavonoid content (TFC) assay

The outcomes obtained in the quantitative analysis of flavonoids are presented in Table 4.1. The amounts of TFC was 42.5 ± 0.008 mg/g in *C. barometz* leaves ethanolic extracts that was slightly higher than TFC of *V. pubescens* leaves ethanolic extracts, 41.7 ± 0.005 mg/g (with equivalent to quercetin).

### 4.2.1.5 Nitric Oxide assay

Nitric oxide is a highly unstable free radical in the plant. The IC₅₀ of NO of the ethanol extract plants was exhibited in Table 4.1. The IC₅₀ of NO of *C. barometz* leaf was 21.2 ± 0.49 µg/ml (% Inhibition, 58.9%), however; the IC₅₀ of NO of *V. pubescens* leaf was 2.7 ± 0.49 µg/ml (% Inhibition, 57.1%). At the same time, the IC₅₀ of NO of BHT, ascorbic acid, quercetin and gallic acid controls that used for comparisons in this
assay were >25 µg/ml, 17.0 ± 0.54 µg/ml, 1.1 ± 0.53 µg/ml, and 2.8 ± 0.48 µg/ml, respectively.

4.2.1.6 Superoxide anion (O$_2^-$) radical scavenging activity assay

The superoxide anion is toxic reactive oxygen and is generated in the plant metabolism that is harmful to photosynthetic pigment and membrane. The results in Table 4.1 exhibited the IC$_{50}$ of O$_2^-$ in the two plants were more than 25 µg/ml. At the same time, the IC$_{50}$ of O$_2^-$ of BHT, ascorbic acid and quercetin controls that were used in the O$_2^-$ scavenging activity assay were 0, 0 and 5.3 ± 0.080 µg/ml, respectively. On the other hand, the percentage inhibition of O$_2^-$ in V. pubescens leaves ethanolic extract was (15.20 %) higher than the percentage inhibition of O$_2^-$ in C. barometz leaves (9.67 %).
Table 4.1: Antioxidant activities of *C. barometz* and *V. pubescens* leaves (*In vitro*).

<table>
<thead>
<tr>
<th>Assay’s Name/ plants; controls</th>
<th>FRAP (µmol Fe(II)/g)</th>
<th>DPPH IC₅₀ (µg/mL)</th>
<th>TPC value (mg/g) Gallic acid/ extract</th>
<th>TFC value (mg/g) Quercetin/ extract</th>
<th>NO IC₅₀ (µg/mL)</th>
<th>O₂⁻ IC₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. barometz</em> leaves</td>
<td>915.7 ± 0.071</td>
<td>30.1 ± 0.05</td>
<td>1578 ± 0.93</td>
<td>42.5 ± 0.008</td>
<td>21.2 ± 0.49</td>
<td>&gt;25</td>
</tr>
<tr>
<td><em>V. pubescens</em> leaves</td>
<td>723.0 ± 0.030</td>
<td>38.3 ± 0.1</td>
<td>1565 ± 0.93</td>
<td>41.7 ± 0.005</td>
<td>2.7 ± 0.49</td>
<td>&gt;25</td>
</tr>
<tr>
<td>BHT</td>
<td>261.0 ± 0.009</td>
<td>9.1 ± 0.15</td>
<td>402 ± 0.25</td>
<td>_</td>
<td>&gt; 25</td>
<td>0</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>457.7 ± 0.005</td>
<td>4.9 ± 0.11</td>
<td>1511 ± 0.90</td>
<td>_</td>
<td>17.0 ± 0.54</td>
<td>0</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1544.3 ± 0.012</td>
<td>1.8 ± 0.04</td>
<td>693 ± 0.42</td>
<td>_</td>
<td>1.1 ± 0.53</td>
<td>5.3 ± 0.080</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>1774.3 ± 0.002</td>
<td>1.4 ± 0.13</td>
<td>1114 ± 0.67</td>
<td>_</td>
<td>2.8 ± 0.48</td>
<td>_</td>
</tr>
</tbody>
</table>

The oxidants activities data of *C. barometz* and *V. pubescens* leaves compared with BHT (butylated hydroxyltoluene), ascorbic acid, quercetin and gallic acid. The values are expressed as mean ± standard (in triplicate).
4.2.2 MTT assay and cytotoxicity in cell line

The mitochondrial reduction MTT assay is one of the most frequently used to determine cytotoxicity and cell proliferation. When *C. barometz* (*Cb*) and *V. pubescens* (*Vp*) leaves crude ethanol extracts and their fractions (*Cb*F1 to *Cb*F7; *Vp*F1 to *Vp*F9) with varying concentrations assessed for 48 hrs treatment on hepatic human cell line WRL-68 cells, the cell viabilities were measured using the MTT assay. In this study, the effects of crude of plants with fractions showed no cytotoxicity and their IC\textsubscript{50} were more than 100 μmol/ml even at higher concentrations (Salama *et al.*, 2013). The moderate cell viability of *C. barometz* and *V. pubescens* crude ethanol extracts were 36% and 27% respectively, while for *Cb*F2 and *Vp*F3 exhibited 32% and 23% respectively and the highest viability of *Cb*F1 and *Vp*F2 was 39 % and 37% respectively at 200 μg/mL in a dose dependent manner (Figures 4.1 and 4.2). Thus, *Cb*F1 and *Vp*F2 that have a higher viability on WRL 68 were subjected to identify the active compounds using liquid chromatography mass spectrometry (LC-MS).

4.2.3 Identification of active compounds of the plant active fraction

LC-MS was used to identify the active compounds of the higher effective fractions, *Cb*F1 and *Vp*F2. Moreover, their obtained peaks with retention time (RT), molecular weight and molecular formula were identified and investigated based on mass of charge (m/z) (Tables 4.2; 4.3 and Figures 4.3, 4.4, 4.5, 4.6 and 4.7). Our results showed and suggested four identified compounds in *Cb*F1 and five identified compounds in *Vp*F2. In *Cb*F1, the suggested compounds are D-Glucose 203.000 [M+Na]\(^+\), 5-Hydroxymethyl-2-furancarboxaldehyde1 27.000 [M+H]\(^+\), Alternariol 281.100 [M+Na]\(^+\), 24-Methylenecycloartanol 441.200 [M+H]\(^+\). However, in *Vp*F2, the suggested compounds are Caffeic acid 182.100 [M-2H]\(^+\), Quercetin 325.100 [M+Na]\(^+\), Vitexin 433.100 [M+H]\(^+\), Apignenin 271.000 [M+H]\(^+\) and luteoloside 449.000 [M+H]\(^+\).
Figure 4.1: The effect of different concentrations of *C. barometz* fractions (*CbF1; CbF2*) and the crude on the viability of WRL-68 cell line. The Data (in triplicate) were expressed as mean ± SEM.
Figure 4.2: The effect of different concentrations of *V. pubescens* fractions (VpF2; VpF3) and the crude on the viability of WRL-68 cell line. The Data (in triplicate) were expressed as mean ± SEM.
Table 4.2: The identified compounds of *C. barometz* (F1) by LC-MS.

<table>
<thead>
<tr>
<th>No</th>
<th>RT (min)</th>
<th>Name of Identified compounds</th>
<th>Mass of charge (m/z)</th>
<th>Molecular weight (g/mol)</th>
<th>Molecular formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.299</td>
<td>D-glucose</td>
<td>203.000 [M+Na]^+</td>
<td>180.156</td>
<td>C_6H_{12}O_6</td>
</tr>
<tr>
<td>2</td>
<td>0.920</td>
<td>5-Hydroxymethyl-2-furancarboxaldehyde</td>
<td>127.000 [M+H]^+</td>
<td>126.110</td>
<td>C_6H_6O_3</td>
</tr>
<tr>
<td>3</td>
<td>1.054</td>
<td>Alternariol</td>
<td>281.100 [M+Na]^+</td>
<td>258.226</td>
<td>C_{14}H_{10}O_5</td>
</tr>
<tr>
<td>4</td>
<td>1.400</td>
<td>24-Methylenecycloartanol</td>
<td>441.200 [M+H]^+</td>
<td>440.744</td>
<td>C_{31}H_{52}O</td>
</tr>
</tbody>
</table>

RT: Retention Time.

Table 4.3: The identified compounds of *V. pubescens* (F2) by LC-MS.

<table>
<thead>
<tr>
<th>No</th>
<th>RT (min)</th>
<th>Name of Identified compounds</th>
<th>Mass of charge (m/z)</th>
<th>Molecular weight (g/mol)</th>
<th>Molecular formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.338</td>
<td>Caffeic acid</td>
<td>182.100 [M-2H]^+</td>
<td>180.16</td>
<td>C_9H_8O_4</td>
</tr>
<tr>
<td>2</td>
<td>0.849</td>
<td>Quercetin</td>
<td>325.100 [M+Na]^+</td>
<td>302.236</td>
<td>C_{15}H_{10}O_7</td>
</tr>
<tr>
<td>3</td>
<td>1.447</td>
<td>Vitexin</td>
<td>433.100 [M+H]^+</td>
<td>432.38</td>
<td>C_{21}H_{20}O_{10}</td>
</tr>
<tr>
<td>4</td>
<td>1.612</td>
<td>Apigenin</td>
<td>271.000 [M+H]^+</td>
<td>270.237</td>
<td>C_{15}H_{10}O_5</td>
</tr>
<tr>
<td>5</td>
<td>2.894</td>
<td>Luteoloside</td>
<td>449.000 [M+H]^+</td>
<td>448.37</td>
<td>C_{21}H_{20}O_{11}</td>
</tr>
</tbody>
</table>

RT: Retention Time.
Figure 4.3: The Mass spectrum (Triple quad QQQ MS ESI’+) and the identified chemical structures (D-Glucose; 5-Hydroxymethyl-2- furancarboxaldehyde) in C. barometz leaves, F1.
Figure 4.4: The Mass spectrum (Triple quad QQQ MS ESI\(^{+}\)) and the identified chemical structures (Alternariol; 24-Methylene cycloartanol) in *C. barometz* leaves, F1.
**Figure 4.5:** The Mass spectrum (Triple quad QQQ MS ESI⁺) and the identified chemical structures (Caffeic acid; Quercetin) in *V. pubescens* leaves, F2.
Figure 4.6: The Mass spectrum (Triple quad QQQ MS ESI⁺) and the identified chemical structures (Vitexin; Apigenin) in *V. pubescens* leaves, F2.
Figure 4.7: The Mass spectrum (Triple quad QQQ MS ESI\(^+\)) and chemical structure of (luteoloside) identified in V. pubescens leaves, F2.

4.2.4 Gene expression

4.2.4.1 Extraction of RNA

The results of RNA concentrations were above 120 ng/µL, and purity (an A260/280 value of ≥2 and A260/230 value of ≥1.7) that were anticipated values for pure RNA measured by Nano-drop 2000 Spectrophotometer (Waltham, Massachusetts, USA). The result of the agarose gel electrophoresis exhibited two sharp and clear bands of RNA that can be observed in 18S and 28S RNA as presented in Figure 4.8. This outcome indicated and verified the integrity of purified RNA in the experiment.
4.2.4.2 Gene expression profiling

In this study, BCL2 (B-cell lymphoma 2) associated X protein (Bax) and Collagen Type I Alpha 2 (Col1a2) genes expressions were assessed using real time-polymerase chain reaction (RT-PCR) for estimation the molecular response of the human skin fibroblast cell line (Hs27) toward the treatments of crude extract of *C. barometz* leaves (the best effective plant, *in vitro* study). The expression comparisons of selected genes demonstrated the molecular response of the cells toward the extract treatments.

Our results in this experiment were expressed as fold changes comparing with the respective control. In Figure 4.9 shows that the extract of *C. barometz* slightly down-regulated the expression of *Bax* (-0.5 fold), and significantly up-regulated the expression of *Col1a2* (2.13 fold) in fibroblast cells.
Figure 4.9: Gene expression analysis of Hs27 fibroblast cells treated with *C. barometz* leaves ethanol extract, expressed in triplicate as fold changes of *Bax* and *Col1a2* genes of untreated and treated with extract groups. Values were expressed as mean ± SEM. *Differences were significant at the $p<0.05$ level compared to untreated cells.

4.3 *In vivo* study

*In vivo* study of *V. pubescens* and *C. barometz* leaves ethanolic extracts had three evaluation studies that were the acute toxicity, the gastro protective effect and the wound healing potential.

4.3.1 Acute toxicity test

None of treated animals with ethanol extract of *C. barometz* or *V. pubescens* leaves demonstrated any mortality or toxic symptoms in this experiment.

4.3.1.1 Biochemical evaluation of blood-serum

There were no body weight changes or abnormal physiological or behavioural variations of animals in both sex at 2 g/kg and 5 g/kg doses during the 14 days following extract administration as shown in Table 4.4. The biochemical remarks on liver and kidney of SD rats and their weights were normal appeared in comparison to the control vehicle groups as shown in Tables 4.5, 4.6, 4., 4.8, 4.9, 4.10 and 4.11.
**Table 4.4**: Effects of *C. barometz* and *V. pubescens* leaves ethanol extract on the body weight of female and male SD rats in the acute toxicity test.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>1st Day</td>
</tr>
<tr>
<td>Vehicle (10% Tween20)</td>
<td>190.3 ± 10</td>
</tr>
<tr>
<td><em>C. barometz</em> (2 g/kg)</td>
<td>181.3 ± 8.8</td>
</tr>
<tr>
<td><em>C. barometz</em> (5 g/kg)</td>
<td>162.0 ± 9.1</td>
</tr>
<tr>
<td><em>V. pubescens</em> (2 g/kg)</td>
<td>186 ± 8.6</td>
</tr>
<tr>
<td><em>V. pubescens</em> (5 g/kg)</td>
<td>169 ± 4.7</td>
</tr>
</tbody>
</table>

Values expressed as mean ± S.E.M. There are no significant variations between groups. Significant value was observed at *p*<0.05.

**Table 4.5**: Effects of *C. barometz* and *V. pubescens* leaves ethanol extract on the liver and kidney weights of female and male SD rats in the acute toxicity test.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Liver Weight</th>
<th>Kidney Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Vehicle (10% Tween20)</td>
<td>6.3 ± 0.3</td>
<td>6.97 ± 0.1</td>
</tr>
<tr>
<td><em>C. barometz</em> (2 g/kg)</td>
<td>6.5 ± 0.2</td>
<td>6.57 ± 0.3</td>
</tr>
<tr>
<td><em>C. barometz</em> (5 g/kg)</td>
<td>5.27 ± 0.1</td>
<td>6.23 ± 0.2</td>
</tr>
<tr>
<td><em>V. pubescens</em> (2 g/kg)</td>
<td>6.57 ± 0.28</td>
<td>6.85 ± 0.28</td>
</tr>
<tr>
<td><em>V. pubescens</em> (5 g/kg)</td>
<td>5.40 ± 0.13</td>
<td>6.57± 0.30</td>
</tr>
</tbody>
</table>

Values expressed as mean ± S.E.M. There are no significant variations between groups. Significant value was observed at *p*<0.05.
Table 4.6: Effects of *C. barometz* and *V. pubescens* leaves extracts on kidney biochemical parameters in female rats in the acute toxicity test.

<table>
<thead>
<tr>
<th>Dose female</th>
<th>Sodium (mmol/L)</th>
<th>Potassium (mmol/L)</th>
<th>Chloride (mmol/L)</th>
<th>CO₂ (mmol/L)</th>
<th>Anion Gap (mmol/L)</th>
<th>Urea (mmol/L)</th>
<th>Creatinine (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (10% Tween20)</td>
<td>147.3 ± 0.76</td>
<td>5.1 ± 0.18</td>
<td>107.0 ± 0.58</td>
<td>25.27 ± 0.52</td>
<td>21.8 ± 0.60</td>
<td>5.28 ± 0.18</td>
<td>38.5 ± 2.73</td>
</tr>
<tr>
<td><em>C. barometz</em> (2 g/kg)</td>
<td>147.00 ± 0.52</td>
<td>4.8 ± 0.09</td>
<td>106.67 ± 0.71</td>
<td>23.47 ± 0.58</td>
<td>21.83 ± 0.79</td>
<td>9.70 ± 0.98</td>
<td>38.83 ± 3.49</td>
</tr>
<tr>
<td><em>C. barometz</em> (5 g/kg)</td>
<td>146.00 ± 0.93</td>
<td>4.8 ± 0.2</td>
<td>107.83 ± 0.79</td>
<td>21.97 ± 1.31</td>
<td>20.67 ± 0.76</td>
<td>6.07 ± 0.39</td>
<td>36.53 ± 3.03</td>
</tr>
<tr>
<td><em>V. pubescens</em> (2 g/kg)</td>
<td>147.3 ± 0.42</td>
<td>4.9 ± 0.09</td>
<td>107.3 ± 0.33</td>
<td>25.45 ± 0.38</td>
<td>21.0 ± 0.52</td>
<td>5.88 ± 0.11</td>
<td>44.7 ± 3.17</td>
</tr>
<tr>
<td><em>V. pubescens</em> (5 g/kg)</td>
<td>146.3 ± 0.71</td>
<td>5.0 ± 0.22</td>
<td>108.5 ± 0.62</td>
<td>23.72 ± 0.41</td>
<td>21.0 ± 0.52</td>
<td>5.55 ± 0.10</td>
<td>37.8 ± 3.28</td>
</tr>
</tbody>
</table>

Values expressed as mean ± S.E.M. There are no significant changes between groups.
Significant value was observed at p<0.05.
Table 4.7: Effects of *C. barometz* and *V. pubescens* leaves extracts on kidney biochemical parameters in male rats in the acute toxicity test.

<table>
<thead>
<tr>
<th>Dose male</th>
<th>Sodium (mmol/L)</th>
<th>Potassium (mmol/L)</th>
<th>Chloride (mmol/L)</th>
<th>CO₂ (mmol/L)</th>
<th>Anion Gap (mmol/L)</th>
<th>Urea (mmol/L)</th>
<th>Creatinine (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (10% Tween20)</td>
<td>146.5 ± 0.76</td>
<td>5.3 ± 0.06</td>
<td>106.0 ± 0.52</td>
<td>26.97 ± 0.39</td>
<td>20.2 ± 0.31</td>
<td>5.33 ± 0.18</td>
<td>32.7 ± 2.58</td>
</tr>
<tr>
<td><em>C. barometz</em> (2 g/kg)</td>
<td>145.67 ± 0.84</td>
<td>5.03 ± 0.15</td>
<td>105.17 ± 1.11</td>
<td>26.07 ± 0.70</td>
<td>19.50 ± 0.43</td>
<td>19.50 ± 0.43</td>
<td>30.50 ± 1.52</td>
</tr>
<tr>
<td><em>C. barometz</em> (5 g/kg)</td>
<td>142.83 ± 0.40</td>
<td>5.40 ± 0.08</td>
<td>101.50 ± 0.34</td>
<td>27.75 ± 0.29</td>
<td>19.00 ± 0.52</td>
<td>5.05 ± 0.15</td>
<td>30.33 ± 1.74</td>
</tr>
<tr>
<td><em>V. pubescens</em> (2 g/kg)</td>
<td>146.2 ± 0.48</td>
<td>5.3 ± 0.15</td>
<td>106.8 ± 1.11</td>
<td>26.58 ± 0.48</td>
<td>20.0 ± 0.26</td>
<td>5.33 ± 0.22</td>
<td>31.2 ± 1.78</td>
</tr>
<tr>
<td><em>V. pubescens</em> (5 g/kg)</td>
<td>144.7 ± 0.49</td>
<td>5.5 ± 0.05</td>
<td>105.7 ± 0.42</td>
<td>26.35 ± 0.42</td>
<td>19.7 ± 0.49</td>
<td>5.12 ± 0.16</td>
<td>31.8 ± 1.35</td>
</tr>
</tbody>
</table>

CO₂: carbon dioxide. Values expressed as mean ± S.E.M. There are no significant changes between groups. Significant value was observed at p<0.05.
Table 4.8: Effects of *C. barometz* and *V. pubescens* leaves extracts on liver biochemical parameters in female rats in the acute toxicity test.

<table>
<thead>
<tr>
<th>Dose female</th>
<th>Total Protein (g/L)</th>
<th>Albumin (g/L)</th>
<th>Globulin (g/L)</th>
<th>TB (μmol/L)</th>
<th>CB (μmol/L)</th>
<th>ALP (IU/L)</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
<th>GGT (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (10% Tween20)</td>
<td>80.3 ± 2.29</td>
<td>14.3 ± 0.67</td>
<td>66.67 ± 1.91</td>
<td>2.7 ± 0.33</td>
<td>1.5 ± 0.2</td>
<td>108.8 ± 9.13</td>
<td>51.0 ± 0.58</td>
<td>216.7 ± 11.72</td>
<td>5.7 ± 0.84</td>
</tr>
<tr>
<td><em>C. barometz</em> (2 g/kg)</td>
<td>80.5 ± 3.16</td>
<td>13.7 ± 0.92</td>
<td>67.50 ± 2.26</td>
<td>1.00 ± 0.67</td>
<td>1 ± 0.00</td>
<td>102.8 ± 10.28</td>
<td>41.3 ± 1.09</td>
<td>206.5 ± 14.86</td>
<td>5.33 ± 0.95</td>
</tr>
<tr>
<td><em>C. barometz</em> (5 g/kg)</td>
<td>77.8 ± 2.18</td>
<td>12.5 ± 0.92</td>
<td>64.67 ± 1.36</td>
<td>1.05 ± 0.97</td>
<td>1 ± 0.00</td>
<td>88.5 ± 13.87</td>
<td>37.3 ± 1.91</td>
<td>194.2 ± 11.79</td>
<td>4.51 ± 0.81</td>
</tr>
<tr>
<td><em>V. pubescens</em> (2 g/kg)</td>
<td>81.2 ± 3.45</td>
<td>13.2 ± 0.87</td>
<td>62.7 ± 1.48</td>
<td>2.2 ± 0.17</td>
<td>1 ± 0.00</td>
<td>108.17 ± 4.33</td>
<td>47.0 ± 1.37</td>
<td>204.2 ± 4.90</td>
<td>5.0 ± 0.52</td>
</tr>
<tr>
<td><em>V. pubescens</em> (5 g/kg)</td>
<td>79.8 ± 1.68</td>
<td>12.7 ± 0.71</td>
<td>64.5 ± 1.12</td>
<td>2.5 ± 0.22</td>
<td>1.5 ± 0.2</td>
<td>105.0 ± 10.33</td>
<td>51.0 ± 1.06</td>
<td>205.7 ± 10.10</td>
<td>4.0 ± 0.37</td>
</tr>
</tbody>
</table>

TB: total bilirubin; CB: conjugated bilirubin; AP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: G-glutamyltransferase. Values expressed as mean ± S.E.M. There are no significant changes between groups. Significant value was observed at p<0.05.
Table 4.9: Effects of *C. barometz* and *V. pubescens* leaves extracts on liver biochemical parameters in male rats in the acute toxicity test.

<table>
<thead>
<tr>
<th>Dose male</th>
<th>Total Protein (g/L)</th>
<th>Albumin (g/L)</th>
<th>Globulin (g/L)</th>
<th>TB (μmol/L)</th>
<th>CB (μmol/L)</th>
<th>ALP (IU/L)</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
<th>GGT (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (10% Tween20)</td>
<td>68.0 ± 1.26</td>
<td>12.2 ± 0.48</td>
<td>56.2 ± 0.75</td>
<td>2.5 ± 0.43</td>
<td>1.7 ± 0.21</td>
<td>196.3 ± 9.75</td>
<td>58.3 ± 4.25</td>
<td>227.3 ± 12.52</td>
<td>3.8 ± 0.31</td>
</tr>
<tr>
<td><em>C. barometz</em> (2 g/kg)</td>
<td>69.00 ± 1.37</td>
<td>11.4 ± 0.61</td>
<td>57.67 ± 1.12</td>
<td>2.00 ± 0.82</td>
<td>0.67 ± 0.33</td>
<td>190.2 ± 18.24</td>
<td>57.83 ± 1.60</td>
<td>216.83 ± 9.82</td>
<td>2.50 ± 1.15</td>
</tr>
<tr>
<td><em>C. barometz</em> (5 g/kg)</td>
<td>68.33 ± 0.88</td>
<td>11.5 ± 0.50</td>
<td>56.83 ± 0.87</td>
<td>1.83 ± 0.79</td>
<td>0.67 ± 0.33</td>
<td>192.2 ± 16.25</td>
<td>58.00 ± 1.81</td>
<td>213.50 ± 6.45</td>
<td>2.40 ± 1.12</td>
</tr>
<tr>
<td><em>V. pubescens</em> (2 g/kg)</td>
<td>69.5 ± 1.65</td>
<td>11.8 ± 0.60</td>
<td>58.2 ± 1.11</td>
<td>2.3 ± 0.21</td>
<td>1.00 ± 0.00</td>
<td>188.33 ± 1.89</td>
<td>56.8 ± 0.70</td>
<td>222.2 ± 7.64</td>
<td>3.7 ± 0.42</td>
</tr>
<tr>
<td><em>V. pubescens</em> (5 g/kg)</td>
<td>67.7 ± 0.88</td>
<td>12.0 ± 0.37</td>
<td>56.0 ± 0.77</td>
<td>2.5 ± 0.22</td>
<td>1.3 ± 0.21</td>
<td>193.2 ± 14.46</td>
<td>56.7 ± 0.84</td>
<td>213.3 ± 5.17</td>
<td>3.3 ± 0.21</td>
</tr>
</tbody>
</table>

TB: total bilirubin; CB: conjugated bilirubin; AP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: G-glutamyltransferase. Values expressed as mean ± S.E.M. There are no significant changes between groups. Significant value was observed at p<0.05.
**Table 4.10**: Effects of *C. barometz* and *V. pubescens* leaves ethanol extract on lipid profile biochemical parameters in female rats in the acute toxicity test.

<table>
<thead>
<tr>
<th>Dose female</th>
<th>Triglyceride (mmol/L)</th>
<th>Total Cholesterol (mmol/L)</th>
<th>HDL Cholesterol (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (10% Tween20)</td>
<td>0.4 ± 0.03</td>
<td>1.92 ± 0.07</td>
<td>1.94 ± 0.05</td>
</tr>
<tr>
<td><em>C. barometz</em> (2 g/kg)</td>
<td>0.30 ± 0.04</td>
<td>0.34 ± 0.72</td>
<td>1.47 ± 0.18</td>
</tr>
<tr>
<td><em>C. barometz</em> (5 g/kg)</td>
<td>0.38 ± 0.07</td>
<td>0.55 ± 0.83</td>
<td>1.59 ± 0.20</td>
</tr>
<tr>
<td><em>V. pubescens</em> (2 g/kg)</td>
<td>0.4 ± 0.04</td>
<td>1.77 ± 0.08</td>
<td>1.86 ± 0.04</td>
</tr>
<tr>
<td><em>V. pubescens</em> (5 g/kg)</td>
<td>0.4 ± 0.03</td>
<td>1.83 ± 0.11</td>
<td>1.93 ± 0.08</td>
</tr>
</tbody>
</table>

Values expressed as mean ± S.E.M. There are no significant changes between groups. Significant value at *p* < 0.05.

**Table 4.11**: Effects of *C. barometz* and *V. pubescens* leaves ethanol extract on lipid profile biochemical parameters in male rats in the acute toxicity test.

<table>
<thead>
<tr>
<th>Dose male</th>
<th>Triglyceride (mmol/L)</th>
<th>Total Cholesterol (mmol/L)</th>
<th>HDL Cholesterol (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (10% Tween20)</td>
<td>0.52 ± 0.04</td>
<td>1.75 ± 0.04</td>
<td>1.69 ± 0.07</td>
</tr>
<tr>
<td><em>C. barometz</em> (2 g/kg)</td>
<td>0.50 ± 0.07</td>
<td>1.57 ± 0.04</td>
<td>1.40 ± 0.05</td>
</tr>
<tr>
<td><em>C. barometz</em> (5 g/kg)</td>
<td>0.40 ± 0.04</td>
<td>1.62 ± 0.09</td>
<td>1.54 ± 0.09</td>
</tr>
<tr>
<td><em>V. pubescens</em> (2 g/kg)</td>
<td>0.54 ± 0.03</td>
<td>1.57 ± 0.02</td>
<td>1.67 ± 0.06</td>
</tr>
<tr>
<td><em>V. pubescens</em> (5 g/kg)</td>
<td>0.44 ± 0.03</td>
<td>1.72 ± 0.07</td>
<td>1.62 ± 0.05</td>
</tr>
</tbody>
</table>

Values expressed as mean ± S.E.M. There are no significant changes between groups. Significant value at *p* < 0.05.
4.3.1.2 **Histological evaluation of the liver and kidney**

The histological observations on the liver and kidney using Haematoxylin and Eosin (H & E) staining were normal in comparison to the control groups of SD rats as shown in Figures 4.10 and 4.11. Male and female rats did not exhibit any significant signs of toxicity at 2 g/kg and 5 g/kg doses.

**Figure 4.10:** Histological sections of the liver and kidney for the acute toxicity experiment. Rats treated with 5 mL/kg of the vehicle (10% Tween20) (A and B). Rats treated with 2 g/kg (2 mL/kg) of the *C. barometz* leaves extract (C and D). Rats treated with 5 g/kg (5 mL/kg) of the *C. barometz* leaves extract (E and F). No significant changes in the structures of the livers A, C, E and kidneys B, D, F between the treated and control groups (H &E stain).
Figure 4.11: Histological sections of the liver and kidney for the acute toxicity experiment. Rats treated with 5 mL/kg of the vehicle (10% Tween 20) (A and B). Rats treated with 2 g/kg (2 mL/kg) of the *V. pubescens* leaves extract (C and D). Rats treated with 5 g/kg (5 mL/kg) of the *V. pubescens* leaves extract (E and F). No significant changes in the structures of the livers A, C, E and kidneys B, D, F between the treated and control groups (H &E stain).
4.3.2 Gastro protective effect study

4.3.2.1 Measurement of mucus content and gastric juice acidity

As the results indicated in Table 4.5, the ulcer SD rats group G2 produced the lowest mucus content of gastric mucosa in the experiment due to the induction of gastric ulcer in rats using the ethanol that was a necrotizing agent. While animal groups pre-treated with *C. barometz* and *V. pubescens* leaves G6 (250 mg/kg) and G7 (500 mg/kg) showed a significant increase in the mucus weight (g) with respect to the ulcer control group G2. Whereas pre-treatment with *C. barometz* leaves, G4 to G7 produced a significant increase in the acidity of the gastric contents compared to G2. Otherwise, the acidity of the gastric contents in SD rats that pre-treated with *V. pubescens* leaves G4 to G7 showed a non-significant increase in respect to ulcer group.

4.3.2.2 Gross gastric lesion evaluation

The effect of *C. barometz* and *V. pubescens* leaves ethanolic extracts on the ulcer area due to ethanol are shown in Table 4.12 and Figures 4.12 and 4.13. The results revealed that pre-treatment of SD rats with *C. barometz* and *V. pubescens* leaves significantly (*p < 0.05*) diminished the ulcer area as compared to the ulcer group G2. The highest dose of *C. barometz* leaves at 500 mg/kg (126.0 ± 5.50 mm²) showed 84.28% inhibition compared to the value recorded from the ulcer control group animals (801.60 ± 35.65 mm²). And the inhibition percentage of the ulcer area in the pre-treated rats with *C. barometz* leaves increased also at doses of 62.5 mg/kg (256.80 ± 32.61 mm²), 125 mg/kg (163.20 ± 22.82 mm²), and 250 mg/kg (150.00 ± 21.39 mm²) and by 67.96%, 79.64% and 81.29%, respectively. The data also revealed an elevation of the inhibition percentage of the ulcer area in the pre-treated rats with *V. pubescens* leaves ethanol extracts at 62.5 mg/kg (336.10 ± 23.22 mm²), 125 mg/kg (319.57 ± 37.02 mm²),
250 mg/kg (180.00 ± 20.87 mm$^2$), 500 mg/kg (166.80 ± 35.05 mm$^2$) doses by 58.09% and 60.13%, 77.55%, and 79.19% correspondingly as compared to ulcer group.
Table 4.12: Effect of *C. barometz* and *V. pubescens* leaves extracts on acidity of gastric content, mucus weight, ulcer area, and inhibition % of ulcer area in stomach.

<table>
<thead>
<tr>
<th>Group</th>
<th>No of group</th>
<th>Pretreatment 5 ml/kg</th>
<th>Post-treatment 5 ml/kg</th>
<th>Mucus Weight (g)</th>
<th>Acidity</th>
<th>Ulcer Area (mm²)</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>G1</td>
<td>10% Tween20</td>
<td>10% Tween20</td>
<td>2.29 ± 0.15</td>
<td>7.17 ± 0.38</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ulcer</td>
<td>G2</td>
<td>10% Tween20</td>
<td>10% Tween20</td>
<td>0.76 ± 0.15</td>
<td>2.77 ± 0.28</td>
<td>801.60 ± 35.65</td>
<td>-</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>G3</td>
<td>20 mg/kg</td>
<td>Absolute ethanol</td>
<td>1.93 ± 0.06*</td>
<td>5.72 ± 0.49*</td>
<td>96.00 ± 20.42*</td>
<td>88.02</td>
</tr>
<tr>
<td></td>
<td>G4-C</td>
<td>(62.5 mg/kg)</td>
<td>Absolute ethanol</td>
<td>0.80 ± 0.03</td>
<td>4.56 ± 0.49*</td>
<td>256.80 ± 32.61*</td>
<td>67.96</td>
</tr>
<tr>
<td></td>
<td>G5-C</td>
<td>(125 mg/kg)</td>
<td>Absolute ethanol</td>
<td>1.32 ± 0.09</td>
<td>4.47 ± 0.16*</td>
<td>163.20 ± 22.82*</td>
<td>79.64</td>
</tr>
<tr>
<td></td>
<td>G6-C</td>
<td>(250 mg/kg)</td>
<td>Absolute ethanol</td>
<td>1.41 ± 0.18*</td>
<td>5.86 ± 0.42*</td>
<td>150.00 ± 21.39*</td>
<td>81.29</td>
</tr>
<tr>
<td></td>
<td>G7-C</td>
<td>(500 mg/kg)</td>
<td>Absolute ethanol</td>
<td>1.78 ± 0.17*</td>
<td>5.20 ± 0.47*</td>
<td>126.00 ± 5.50*</td>
<td>84.28</td>
</tr>
<tr>
<td></td>
<td>G4-V</td>
<td>(62.5 mg/kg)</td>
<td>Absolute ethanol</td>
<td>0.79 ± 0.05</td>
<td>4.99 ± 0.53</td>
<td>336.10 ± 23.22*</td>
<td>58.09</td>
</tr>
<tr>
<td></td>
<td>G5-V</td>
<td>(125 mg/kg)</td>
<td>Absolute ethanol</td>
<td>0.83 ± 0.05</td>
<td>4.73 ± 0.70</td>
<td>319.57 ± 37.02*</td>
<td>60.13</td>
</tr>
<tr>
<td></td>
<td>G6-V</td>
<td>(250 mg/kg)</td>
<td>Absolute ethanol</td>
<td>1.47 ± 0.20*</td>
<td>4.94 ± 0.49</td>
<td>180.00 ± 20.87*</td>
<td>77.55</td>
</tr>
<tr>
<td></td>
<td>G7-V</td>
<td>(500 mg/kg)</td>
<td>Absolute ethanol</td>
<td>1.80 ± 0.19*</td>
<td>4.75 ± 0.65</td>
<td>166.80 ± 35.05*</td>
<td>79.19</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± SEM. * Indicates significance at p< 0.05 compared to ulcer group.
Figure 4.12 Effect of *C. barometz* leaves on the macroscopic appearance of the gastric mucosa in ethanol-induced gastric mucosal lesions in male SD rats. G1 (vehicle group) showed no injuries to the gastric mucosa, G2 (ulcer control group) had severe injuries in the gastric mucosa, G3 (omeprazole) showed mild disruptions of the surface epithelium in the gastric mucosa. G4 (62.5 mg/kg), G5 (125 mg/kg), G6 (250 mg/kg), G7 (500 mg/kg) *C. barometz* extract had moderate and mild disruptions of the surface epithelium in the gastric mucosa in dependent of dose manner. White arrow points to the haemorrhagic bands.
Figure 4.12: Effect of *V. pubescens* leaves on the macroscopic appearance of the gastric mucosa in ethanol-induced gastric mucosal lesions in male SD rats. G1 (vehicle group) showed no injuries to the gastric mucosa, G2 (ulcer control group) had severe injuries in the gastric mucosa, G3 (omeprazole) showed mild disruptions of the surface epithelium in the gastric mucosa. G4 (62.5mg/kg), G5 (125 mg/kg), G6 (250 mg/kg), G7 (500 mg/kg) *V. pubescens* extract had moderate and mild disruptions of the surface epithelium in the gastric mucosa in dependent of dose manner. White arrow points to the haemorrhagic bands.
4.3.2.3 Histological gastric lesions evaluation

i. H & E staining

Histological observation exhibited the gastric mucosa damage extensively in the G2 ulcer control group of male SD rats. Furthermore, there were necrotic lesions in the deep gastric mucosa that revealed extensive leucocytes infiltration and edema of the submucosal layer as illustrated in Figures 4.14 and 4.15. Otherwise, the pre-treated SD rats with *C. barometz* and *V. pubescens* leaves ethanolic extracts in the G4 to G7 groups showed comparatively an improvement protection of the gastric mucosa with a depression in or absence of leucocytes infiltration and edema in Figures 4.14 and 4.15. The extracts manifested protective effects in a dose-dependent manner.
Figure 4.13: Effect of *C. barometz* leaves on the histology (H and E staining) of ethanol-induced gastric mucosa damage in male SD rats. G1. 10% Tween 20 (Vehicle control) has intact surface mucosal epithelium, no lesion. G2. 10% Tween 20 (ulcer control) has severe disruption of the surface epithelium and necrotic lesions and extensive edema of the submucosal layer and leukocyte infiltration. G3. Omeprazole (20 mg/kg) has mild disruption of the surface epithelium mucosa, and there is a reduction in the submucosal edema and leukocyte infiltration. G4. *C. barometz* (62.5 mg/kg) has moderate disruption. G5. *C. barometz* (125 mg/kg) has a moderate to mild disruption of the surface epithelium. G6. *C. barometz* (250 mg/kg) has mild disruption of the surface epithelium. G7. *C. barometz* (500 mg/kg) has mild disruption of the surface epithelium. For ulcer area (White arrow), for submucosal edema and leukocyte infiltration (blue arrow).
Figure 4.14: Effect of *V. pubescens* leaves on the histology (H and E staining) of ethanol-induced gastric mucosa damage in male SD rats. G1. 10% Tween20 (Vehicle control) has intact surface mucosal epithelium, no lesion. G2. 10% Tween 20 (ulcer control) has severe disruption of the surface epithelium and necrotic lesions and extensive edema of the submucosal layer and leukocyte infiltration. G3. Omeprazole (20 mg/kg) has mild disruption of the surface epithelium mucosa, and there is a reduction in the submucosal edema and leucocyte infiltration. G4. *V. pubescens* (62.5 mg/kg) has moderate disruption. G5. *V. pubescens* (125 mg/kg) has a moderate to mild disruption of the surface epithelium. G6. *V. pubescens* (250 mg/kg) has mild disruption of the surface epithelium. G7. *V. pubescens* (500 mg/kg) has mild disruption of the surface epithelium. For ulcer area (White arrow), for submucosal edema and leucocyte infiltration (blue arrow).
ii. Mucosal glycoprotein (PAS) staining

The existence of glycoprotein was demonstrated by the presence of a magenta coloration in PAS staining of the gastric mucosal cell layer in G1 vehicle (normal control) group as illustrated in Figures 4.16 and 4.17. The gastric mucosa of the pre-treated rats with *C. barometz* and *V. pubescens* leaves ethanolic extracts showed a gradual increase in PAS staining intensity compared to the ulcer control group G2 in a dose-dependent manner (Figures 4.16 and 4.17). The accumulation of the magenta colour in the mucosal cell layer was an indicator of increasing in the PAS staining intensity and higher glycoprotein content. Nevertheless, this magenta colour decreased and observed to be not plentiful in the gastric mucosa of the ulcer group where the ulcer was induced with ethanol.
Figure 4.15: Effect of *C. barometz* leaves on gastric tissue glycoprotein PAS staining in ethanol-induced gastric ulcers in SD rats. G1 (vehicle group) had accumulation of the magenta colour of PAS intensity in the mucosal cells layer; G2 (ulcer control group) that had decrease and not abundantly observed of a magenta color of PAS intensity, G3 (omeprazole), G4 (62.5 mg/kg), G5 (125 mg/kg), G6 (250 mg/kg), and G7 (500 mg/kg) of *C. barometz* leaves showed an increase in PAS staining intensity by the accumulation of magenta colour in the mucosal cells layer compared to the ulcer group in a dose dependent manner. Red arrow indicates the PAS staining of glycoprotein.
Figure 4.16: Effect of *V. pubescens* leaves on gastric tissue glycoprotein PAS staining in ethanol-induced gastric ulcers in SD rats. G1 (vehicle group) had accumulation of the magenta colour of PAS intensity in the mucosal cells layer; G2 (Ulcer control group) that had decrease and not abundantly observed of a magenta color of PAS intensity, G3 (omeprazole), G4 (62.5 mg/kg), G5 (125 mg/kg), G6 (250 mg/kg), and G7 (500 mg/kg) of *V. pubescens* leaves showed an increase in PAS staining intensity by the accumulation of magenta colour in the mucosal cells layer compared to the ulcer group in a dose dependent manner. Red arrow indicates the PAS staining of glycoprotein.
iii. Immunohistochemistry staining

In the immunohistochemical antigen staining, the expression level of Heat Shock protein 70 (HSP70) protein in the gastric mucosa indicated reduced expression in the ulcer control group G2; however, over-expression (brown coloration) of HSP70 protein appeared in rats pre-treated with omeprazole G3 or *C. barometz* or *V. pubescens* leaves extracts (G4 to G7) in a dependent dose manner (Figures 4.18 and 4.19). Conversely, the immunohistochemistry staining of Bcl-2-associated X protein (Bax) protein in the gastric wall mucosa showed over-expression (brown coloration) in the ulcer control group while reduced expression was observed in rats pre-treated with omeprazole or plant extracts in a dependent dose manner (Figures 4.20 and 4.21).
Figure 4.17: Immunohistochemistry analysis of HSP70 expression of C. barometz leaves in the gastric mucosa of SDale rats. G1 (vehicle group), G2 (Ulcer control group), G3 (omeprazole), G4 (62.5 mg/kg), G5 (125 mg/kg), G6 (250 mg/kg) and G7 (500 mg/kg). C. barometz extract. HSP70 protein expression was up regulated in rats pre-treated with C. barometz in a dose dependent manner and with omeprazole. Down-regulation appeared in the ulcer control group. Yellow arrow indicates the brown stain of HSP70.
Figure 4.18: Immunohistochemistry analysis of HSP70 expression of *V. pubescens* leaves in the gastric mucosa of SD ale rats. G1 (vehicle group), G2 (Ulcer control group), G3 (omeprazole), G4 (62.5 mg/kg), G5 (125 mg/kg), G6 (250 mg/kg) and G7 (500 mg/kg) *V. pubescens* extract. HSP70 protein expression was up-regulated in rats pre-treated with *V. pubescens* in a dose dependent manner and with omeprazole. Down-regulation appeared in the ulcer control group. Yellow arrow indicates the brown stain of HSP70.
Figure 4.19: Immunohistochemistry analysis of the expression of the Bax protein of *C. barometz* leaves in the gastric mucosa of SD male rats. G1 (vehicle group), G2 (ulcer control group), G3 (omeprazole), G4 (62.5 mg/kg), G5 (125 mg/kg), G6 (250 mg/kg) and G7 (500 mg/kg) *C. barometz* extract. Bax protein expression was down regulated in rats pre-treated with *C. barometz* in a dependent dose manner and with omeprazole though up regulated in the ulcer control group. Orange arrow indicates the stain of Bax protein.
Figure 4.20: Immunohistochemistry analysis of the expression of the Bax protein of *V. pubescens* leaves in the gastric mucosa of SD male rats. G1 (vehicle group), G2 (Ulcer control group), G3 (omeprazole), G4 (62.5 mg/kg), G5 (125 mg/kg), G6 (250 mg/kg) and G7 (500 mg/kg) *V. pubescens* extract. Bax protein expression was down regulated in rats pre-treated with *V. pubescens* in a dependent dose manner and with omeprazole though up-regulated in the ulcer control group. Orange arrow indicates the stain of Bax protein.
4.3.2.4 Measurements of antioxidant activities and lipid peroxidation (MDA) levels of gastric tissue homogenates

The parameters of antioxidant activities that were superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) with lipid peroxidation levels of malondialdehyde (MDA) were evaluated in the gastric homogenates of all SD rats in each group in this study. The ulcer group of rats revealed a major reduction of antioxidants SOD, CAT and GPx activities of the gastric homogenate among groups when compared with vehicle control group. Even though, the pre-treatment groups with C. barometz and V. pubescens ethanol extracts or with omeprazole demonstrated elevation of SOD, CAT and GPx activities with respect to the ulcer control group as shown in Figure 4.22 and 4.23. The SOD enzyme activities were significant high of each pre-treated rats with each plant in the groups G6 and G7 at doses 250 mg/kg and 500 mg/kg respectively. The CAT enzyme activities increased significantly in all groups when compared to G2 (ulcer control group) except G6 (at 250 mg/kg dose) of pre-treated with V. pubescens had a non-significant increase. There was significant increase of GPx enzyme activities for gastric mucosal homogenates in pre-treated animals with four doses of C. barometz and V. pubescens leaves extracts and omeprazole in respect to G2. However, the MDA of four doses groups of C. barometz and V. pubescens leaves extracts as well as omeprazole group were significantly lower than ulcer control group G2 as illustrated in Figure 4.22 and 4.23.
Figure 4.22: Effect of *C. barometz* ethanol extract on gastric tissue homogenate of superoxide dismutase (SOD) (A); catalase (CAT) (B); glutathione peroxidase (GPx) (C); malondialdehyde (MDA) assays (D). G1 (vehicle group), G2 (ulcer control group), G3 (omeprazole), G4 (62.5 mg/kg), G5 (125 mg/kg), G6 (250 mg/kg) and G7 (500 mg/kg) of *C. barometz* doses of ethanol extracts. All values (in triplicate) are expressed as mean ± SEM. * significance was at *p*<0.05 compared to ulcer group.
Figure 4.23: Effect of *V. pubescens* ethanol extract on gastric tissue homogenate of superoxide dismutase (SOD) (A); catalase (CAT) (B); glutathione peroxidase (GPx) (C); malondialdehyde (MDA) assays (D). G1 (vehicle group), G2 (ulcer control group), G3 (omeprazole), G4 (62.5 mg/kg), G5 (125 mg/kg), G6 (250 mg/kg) and G7 (500 mg/kg) of *V. pubescens* doses of ethanol extracts. All values (in triplicate) are expressed as mean ± SEM. * significance was at $p<0.05$ compared to ulcer group.
### 4.3.3 Wound healing potential study

#### 4.3.3.1 Wound healing potential in experimental normal and diabetic rats

The wound healing of the *C. barometz* and *V. pubescens* leaves were assessed grossly in normal (non-diabetic) and diabetic male SD rats as depicted in Figures 4.24, 4.25, 4.26, 4.27 and 4.28.

In the dressed wounds of normal (non-diabetic) SD rats with Intrasite gel (reference groups) demonstrated remarkable acceleration rate of wound closure in the skin (87%) compared to vehicle group (CMC 2%) (72%) in this experiment as shown in Figure 4.30. In addition, Intrasite gel induced a significant decrease in the wound area on the 5th and 10th days (159 ± 13.9 mm$^2$ and 41 ± 3.2 mm$^2$) when compared with vehicle group (89 ± 5.9 mm$^2$). The wound closure area’s estimation of dressed wounds with 0.2 mL of 100 mg/mL of *C. barometz*, 200 mg/mL of *C. barometz* and 100 mg/mL of *V. pubescens* at 10th day post excision revealed a significant reduction (43± 2.7 mm$^2$, 28 ± 2.3 mm$^2$ and 38 ± 2.6 mm$^2$, respectively) when compared with vehicle group as shown in Figure 4.29. Conversely, there were marked healing acceleration of dressed wounds with 0.2 mL of 100 mg/mL of *C. barometz*, 200 mg/mL of *C. barometz* and 200 mg/mL of *V. pubescens* at 10th day post excision and exhibited significant increase of wound closure percentage (86%, 91% and 85%, respectively) with respect to vehicle group.

On the other hand, the wound healing in the diabetic patients is characterized by extended periods of healing, slower repair and extensive scarring. In this study, the visual inspection of the wound area in diabetic rats showed a significant reduction in wound area after wound dressing with 100 mg/mL of *C. barometz*, 200 mg/mL of *C. barometz* and 100 mg/mL of *V. pubescens* and 100 mg/mL of *V. pubescens* within 15 days post excision (62.0 ± 2.5 mm$^2$, 44.7 ± 3.6 mm$^2$, 68.0 ± 4.6 mm$^2$ and 54 ± 3.4 mm$^2$, respectively) comparable to vehicle group (104 ± 4.6 mm$^2$) (Figure 4.31). Moreover, the dressed wounds
with 0.2 ml of Intrasite gel in diabetic rats had a significant decrease of wound areas at 15th day of healing (48 ± 1.9 mm²) than vehicle (CMC 2%) group. These outcomes were further substantiated by the observed increase in wound closure percentage with increasing treatment concentrations of two plants in diabetic wounds on day 15 post excision as illustrated in Figure 4.32. They have marked accelerations of dressed wounds with 0.2 mL of 100 mg/mL of *C. barometz*, 200 mg/mL of *C. barometz* and 100 mg/mL of *V. pubescens* and 200 mg/mL of *V. pubescens* at 15th day post excision (80%, 86%, 78% and 83%, respectively) with respect to vehicle group (67%) as shown in Figure 4.32.

The above evaluations provide further independent confirmation that *C. barometz* and *V. pubescens* treatment effectively improves skin wounds in a dose-dependent manner. Furthermore, the above outcomes suggest that a high dose of *C. barometz* showed more effective in improving the progression of wound healing than high dose of *V. pubescens* and Intrasite gel.
Figure 4.21: Effect of *C. barometz* and *V. pubescens* on the gross appearance of wound closure area treatment at day 5-post-wounding in experimental normal SD rats. 0.2 mL of (G1) vehicle group (CMC 2%), (G2) Intrasite gel group (Reference group), (G3) 100 mg/mL of *C. barometz* treated group, (G4) 200 mg/mL of *C. barometz* treated group, (G5) 100 mg/mL of *V. pubescens* treated group, (G6) 200 mg/mL of *V. pubescens*. Grossly, wounds dressed with *C. barometz* and *V. pubescens* or with Intrasite gel group showed considerable signs of dermal healing that healed faster compared to group received the vehicle control (CMC 2%).
Figure 4.22: Effect of *C. barometz* and *V. pubescens* on the gross appearance of wound closure area treatment at day 10-post-wounding in experimental normal SD rats. 0.2 mL of (G1) vehicle group (CMC 2%), (G2) Intrasite gel group (Reference group), (G3) 100 mg/mL of *C. barometz* treated group, (G4) 200 mg/mL of *C. barometz* treated group, (G5) 100 mg/mL of *V. pubescens* treated group, (G6) 200 mg/mL of *V. pubescens*. Grossly, wounds dressed with *C. barometz* and *V. pubescens* or with Intrasit gel group showed considerable signs of dermal healing that healed faster compared to group received the vehicle control (CMC 2%).
Figure 4.23: Effect of *C. barometz* and *V. pubescens* on the gross appearance of wound closure area treatment at day 5-post-wounding in experimental diabetic SD rats. 0.2 mL of (G1) vehicle group (CMC 2%), (G2) Intrasite gel group (Reference group), (G3) 100 mg/mL of *C. barometz* treated group, (G4) 200 mg/mL of *C. barometz* treated group. (G5) 100 mg/mL of *V. pubescens* treated group, (G6) 200 mg/mL of *V. pubescens*. Grossly, wounds dressed with *C. barometz* and *V. pubescens* or with Intrasit gel group showed considerable signs of dermal healing that healed faster compared to group received the vehicle control (CMC 2%).
**Figure 4.24:** Effect of *C. barometz* and *V. pubescens* on the gross appearance of wound closure area treatment at day 10-post-wounding in experimental diabetic SD rats. 0.2 mL of (G1) vehicle group (CMC 2%), (G2) Intrasite gel group (Reference group), (G3) 100 mg/mL of *C. barometz* treated group, (G4) 200 mg/mL of *C. barometz* treated group, (G5) 100 mg/mL of *V. pubescens* treated group, (G6) 200 mg/mL of *V. pubescens*. Grossly, wounds dressed with *C. barometz* and *V. pubescens* or with Intrasite gel group showed considerable signs of dermal healing that healed faster compared to group received the vehicle control (CMC 2%).
**Figure 4.25**: Effect of *C. barometz* and *V. pubescens* on the gross appearance of wound closure area treatment at day 15-post-wounding in experimental diabetic SD rats. 0.2 mL of (G1) vehicle group (CMC 2%), (G2) Intrasite gel group (Reference group), (G3) 100 mg/mL of *C. barometz* treated group, (G4) 200 mg/mL of *C. barometz* treated group. (G5) 100 mg/mL of *V. pubescens* treated group, (G6) 200 mg/mL of *V. pubescens*. Grossly, wounds dressed with *C. barometz* and *V. pubescens* or with Intrasite gel group showed considerable signs of dermal healing that healed faster compared to group received the vehicle control (CMC 2%).
Figure 4.26: Effect of *C. barometz* and *V. pubescens* leaves extracts on wound closure area mm² on 0, 5th and 10th days post wounding in normal SD rats for groups of vehicle (CMC 2%), Intrasite gel, *C. barometz* (200 mg/mL; 100 mg/mL) and *V. pubescens* (200 mg/mL; 100 mg/mL). Data were expressed as mean ± SEM.*Significant value was at P< 0.05 compared with vehicle (CMC 2%) group.

Figure 4.27: Effect of *C. barometz* and *V. pubescens* leaves ethanol extracts on wound closure % on 10th day and 15th day post wounding in normal SD rats for groups of vehicle (CMC 2%), Intrasite gel, *C. barometz* (200 mg/mL; 100 mg/mL) and *V. pubescens* (200 mg/mL; 100 mg/mL). Data were expressed as mean ± SEM.*Significant value was at P< 0.05 compared with vehicle (CMC 2%) group.
**Figure 4.28:** Effect of *C. barometz* and *V. pubescens* leaves extracts on wound closure area mm$^2$ on 0, 10$^{th}$ and 15$^{th}$ days post wounding in diabetic SD rats for groups of vehicle (CMC 2%), Intrasite gel, *C. barometz* (200 mg/mL; 100 mg/mL) and *V. pubescens* (200 mg/mL; 100 mg/mL). Data were expressed as mean ± SEM.*Significant value was at $P<0.05$ compared with vehicle (CMC 2%) group.

**Figure 4.29:** Effect of *C. barometz* and *V. pubescens* leaves ethanol extracts on wound closure % on 5$^{th}$ day, 10$^{th}$ day and 15$^{th}$ day post wounding in diabetic SD rats for groups of vehicle (CMC 2%), Intrasite gel, *C. barometz* (200 mg/mL; 100 mg/mL) and *V. pubescens* (200 mg/mL; 100 mg/mL). Data were expressed as mean ± SEM.*Significant value was at $P<0.05$ compared with vehicle (CMC 2%) group.
4.3.3.2 Histological evaluation of wound tissue

i. Haematoxylin and eosin staining

The wounded tissues of the normal and diabetic rats were evaluated using H&E staining at 10\textsuperscript{th} day (in normal rats) and 15\textsuperscript{th} day (in diabetic rats) as illustrated in Figures 4.33 and 4.34, respectively. The wider wound scars, the granulation tissue with high amounts of inflammatory cell infiltrate, blood vessels formation (angiogenesis) and a few formed collagen fibres were observed in the vehicle group (2\% CMC) due to slower wound healing and the failure of complete wound enclosure than those of the Intrasite gel treated groups. In comparison to the vehicle group, the granulated tissues in the Intrasite gel and treated groups with \textit{C. barometz} and \textit{V. pubescens} had a smaller scar width with less inflammatory cell infiltrate, more fibroblasts, marked blood vessel formation, and increased of collagen deposition.
Figure 4.30: Effect of *C. barometz* and *V. pubescens* on wound healed sections with H & E stain on the day 10 in experimental SD normal rats. (G1) vehicle group (CMC 2%), (G2) Intrasite gel group (Reference group), (G3) 100 mg/mL of *C. barometz* treated group, (G4) 200 mg/mL of *C. barometz* treated group. (G5) 100 mg/mL of *V. pubescens* treated group, (G6) 200 mg/mL of *V. pubescens*. White arrows indicated the wound healing area and re-epithelialization (scar width), GT: granulation tissue, S: scar, D: dermis, and E: epidermis. The figure shows significantly reduction of scar width in groups that dressed with intrasit gel and extract plants accompanied with less inflammatory cell infiltrate, more fibroblast, marked of blood vessel formation and increase of collagen deposition.
Figure 4.31: Effect of *C. barometz* and *V. pubescens* on wound sections stained with H & E on day 15 in experimental diabetic SD rats. (G1) vehicle group (CMC 2%), (G2) Intrasisite group (Reference group), (G3) 100 mg/mL of *C. barometz* treated group, (G4) 200 mg/mL of *C. barometz* treated group. (G5) 100 mg/mL of *V. pubescens* treated group, (G6) 200 mg/mL of *V. pubescens*. White arrows indicated the wound healing area, GT: granulation tissue, S: scar, D: dermis, and E: epidermis. The figure shows significantly reduction of scar width in groups that dressed with intrasit gel and extract plants accompanied with less inflammatory cell infiltrate, more fibroblast, marked of blood vessel formation and increase of collagen deposition.
**ii. Masson’s trichrome staining**

Masson’s trichrome staining of the healed wound tissue at 10th day in normal rats and at 15th day in diabetic rats post incision revealed high collagen accumulation and the generation of new capillary vessels below the endothelial cells in the dressed rats with Intrasite gel when compared to the dressed rats with 2% CMC of vehicle groups as shown in Figures 4.35 and 4.36. The skin sections of wound dressed with 200 mg/mL of *C. barometz* showed extensive collagen accumulation (blue colour), however; skin sections of wound dressed with 100 mg/mL of *C. barometz*, 200 mg/mL of *V. pubescens* and 100 mg/mL of *V. pubescens* showed mild to moderate collagen accumulation with compared to G1.
Figure 4.32: Effect of *C. barometz* and *V. pubescens* on wound healed sections with Masson’s trichrome stain on day 10 in experimental normal SD rats. (G1) vehicle group (CMC 2%), (G2) Intrasite gel group (Reference group), (G3) 100 mg/ml of *C. barometz* treated group, (G4) 200 mg/mL of *C. barometz* treated group, (G5) 100 mg/mL of *V. pubescens* treated group, (G6) 200 mg/mL of *V. pubescens*. White arrows indicated the wound closure area and re-epithelialization (scar width), G: granulation tissue. High collagen accumulation (blue color) and the generation of new capillary vessels below the endothelial cells in the dressed rats with Intrasite gel and plants when compared to the dressed rats with 2% CMC of vehicle groups.
Figure 4.33: Effect of *C. barometz* and *V. pubescens* on wound healed sections with Masson’s trichrome stain on day 15 in experimental diabetic SD rats. (G1) vehicle group (CMC 2%), (G2) Intrasite gel group (Reference group), (G3) 100 mg/ml of *C. barometz* treated group, (G4) 200 mg/mL of *C. barometz* treated group, (G5) 100 mg/mL of *V. pubescens* treated group, (G6) 200 mg/mL of *V. pubescens*. White arrows indicated the wound closure area and re-epithelialization (scar width), G: granulation tissue. High collagen accumulation (blue color) and the generation of new capillary vessels below the endothelial cells in the dressed rats with Intrasite gel and plants when compared to the dressed rats with 2% CMC of vehicle groups.
iii. Immunohistochemistry staining of wound tissue

The immunohistochemistry staining of HSP70 and Bax proteins expressions on the healed wounds of normal and diabetic rats with *C. barometz* and *V. pubescens* leaves are presented in Figures 4.37, 4.38, 4.39 and 4.40, respectively.

The findings of immunohistochemistry of HSP70 staining showed that the vehicle group (2% CMC) had lower HSP70 expression and normal histological examination of the healed skin than the other treated groups. Conversely, the healed skin tissue of Intrasite gel group and the treated groups with *C. barometz* and *V. pubescens* exhibited higher HSP70 protein expression with fewer inflammatory cells and higher fibroblasts when compared with the vehicle group. Furthermore, *C. barometz* (200 mg/mL) showed the highest HSP70 protein expression among the other treated groups.

On the other hand, the results of immunohistochemistry of Bax staining on the healed wounds exhibited that the vehicle groups had greater Bax protein expression than the other groups that received treatment. The histopathological examination of the Intrasite gel groups showed considerably lower Bax expression compared with the vehicle group (2% CMC). Moreover, the tissue sections of healed wounds from the treated groups with *C. barometz* and *V. pubescens* displayed less Bax staining with few inflammatory cells, indicating improvement in the healing when compared to the vehicle groups. Sections of the healed wounds of *C. barometz* (200 mg/mL) showed the lowest Bax protein expression among the other treated groups.
Figure 4.34: Effect of *C. barometz* and *V. pubescens* on wound healed sections with HSP70 stain on day 10 in experimental normal SD rats. (G1) vehicle group (CMC 2%), (G2) Intrasite group (Reference group), (G3) 100 mg/mL of *C. barometz* treated group, (G4) 200 mg/mL of *C. barometz* treated group. (G5) 100 mg/mL of *V. pubescens* treated group, (G6) 200 mg/mL of *V. pubescens*. The immunohistochemistry staining indicated a down-expression of HSP70 protein in the vehicle group G1 and over-expression in the dressed rats with Intrasite gel and plants when compared to the dressed rats with 2% CMC of vehicle groups (Orange arrow: brown color).
Figure 4.35: Effect of *C. barometz* and *V. pubescens* on wound healed sections with HSP70 stain on day 15 in experimental diabetic SD rats. (G1) vehicle group (CMC 2%), (G2) Intrasite group (Reference group), (G3) 100 mg/ml of *C. barometz* treated group, (G4) 200 mg/mL of *C. barometz* treated group, (G5) 100 mg/mL of *V. pubescens* treated group, (G6) 200 mg/mL of *V. pubescens*. Inflammatory cells more, collagen deposition less. The immunohistochemistry staining indicated down-expression of HSP70 protein (orange arrow: brown color) in the vehicle group G1 and an over-expression in G2, G3, G4, G5 and G6.
Figure 4.36: Effect of *C. barometz* and *V. pubescens* on wound healed sections with Bax stain on day 10 in experimental normal SD rats. (G1) vehicle group (CMC 2%), (G2) Intrasite group (Reference group), (G3) 100 mg/ml of *C. barometz* treated group, (G4) 200 mg/mL of *C. barometz* treated group, (G5) 100 mg/mL of *V. pubescens* treated group, (G6) 200 mg/mL of *V. pubescens*. Inflammatory cells less, collagen deposition more. The immunohistochemistry staining indicated over-expression of Bax protein in the vehicle group G1 and down-expression in in the dressed rats with Intrasite gel and plants when compared to the dressed rats with 2% CMC of vehicle groups (blue arrow: brown color).
Figure 4.37: Effect of *C. barometz* and *V. pubescens* on wound healed sections with Bax stain on day 15 in experimental diabetic SD rats. (G1) vehicle group (CMC 2%), (G2) Intrasite group (Reference group), (G3) 100 mg/ml of *C. barometz* treated group, (G4) 200 mg/mL of *C. barometz* treated group, (G5) 100 mg/mL of *V. pubescens* treated group, (G6) 200 mg/mL of *V. pubescens*. Inflammatory cells less, collagen deposition more. The immunohistochemistry staining indicated over-expression of Bax protein in the vehicle group G1 and down-expression in the dressed rats with Intrasite gel and plants when compared to the dressed rats with 2% CMC of vehicle groups (blue arrow: brown colour).
4.3.3.3 Measurements of superoxide dismutase activity (SOD) and lipid peroxidation (MDA) levels of wound tissue homogenates

The results of superoxide dismutase activity (SOD) and malondialdehyde (MDA) evaluations in tissue homogenates of the healed wounds in normal and diabetic rats for *C. barometz* and *V. pubescens* ethanol extracts are shown in Figures 4.41 and 4.42, respectively.

Treatment with either 200 mg/mL dose of *C. barometz*, 100 mg/mL dose of *V. pubescens* and 200 mg/mL dose of *V. pubescens* ethanol extracts of skin tissues in normal rats resulted in a significant (*p*<0.05) increase in SOD enzyme activities when compared to vehicle group. Otherwise, a significant decrease in MDA levels in a dosage dependent fashion in normal rats treated with *C. barometz* and *V. pubescens* that is similar to Intrasite gel comparing to the vehicle group.

On the other hand, the *in vivo* antioxidant enzyme analysis of SOD activity assay revealed significantly (*p*<0.05) accelerated wound healing in treated diabetic rats with *C. barometz* and *V. pubescens* in both doses (100 mg/mL and 200 mg/mL) similar to those with treated Intrasite gel in a dosage dependent manner compared to the vehicle group. However, the dressing using these two plants resulted in a significant decrease in MDA levels of skin tissue in a dosage dependent manner compared to the vehicle group.
Figure 4.38: Effect of *C. barometz* and *V. pubscens* ethanol extracts on wound tissue homogenate of A: superoxide dismutase (SOD) and B: malondialdehyde (MDA) assays in normal rats. G1 (Vehicle CMC 2% group), G2 (Intrasite gel group), G3 (100 mg/mL of *C. barometz* treated group), G4 (200 mg/mL of *C. barometz* treated group), G5 (100 mg/mL of *V. pubscens* treated group) and G6 (200 mg/mL of *V. pubscens* treated group). All values (in triplicate) are expressed as mean ± SEM. * significant at *p*<0.05 compared to the vehicle group.

Figure 4.39: Effect of *C. barometz* and *V. pubscens* ethanol extract on healed wound tissue homogenate of A: superoxide dismutase (SOD) and B: malondialdehyde (MDA) assays in diabetic rats. G1 (Vehicle group), G2 (Intrasite gel group), G3 (100 mg/mL of *C. barometz* treated group), G4 (200 mg/mL of *C. barometz* treated group), G5 (100 mg/mL of *V. pubscens* treated group) and G6 (200 mg/mL of *V. pubscens* treated group). All values (in triplicate) are expressed as mean ± SEM. * significant at *p*<0.05 compared to the vehicle group.
CHAPTER 5: DISCUSSION

5.1 Discussion

Many medications or drugs have various medicinal complications in the treatment of ulcers either gastric or wound (Ji et al., 2012). This might be due to a drug interaction or unpleasant side effects that lead to unsuccessful or not fully efficient to manage and promote the ulcers (Abdelwahab et al., 2013). A huge number of researchers have reported on the numerous remedial plants applied in folk medicine as antiulcer mediators with fewer or milder side effects and contain a wide variety of antioxidants (Hossain & Nagooru, 2011; Chen et al., 2012). Plant extracts are attractive sources of new drugs and have been shown to produce promising results in the treatment of gastric and wounded ulcers. In line with this, *C. barometz* and *V. pubescens* which have been used in traditional medicine as anti-inflammatory plants for treatment many diseases including gastric ulcers and wounds (Zhao et al., 2011; Meena et al., 2011) were investigated. This study was carried out to evaluate the gastro-protective effects and wound healing potential of *C. barometz* and *V. pubescens* leaves.

5.1.1 Antioxidant potency

In the pathological conditions, most of free radicals are produced in the body such as reactive oxygen species (ROS). The destructive effects of ROS create a biological damage in the organ as result of excessive oxidative stress and poor antioxidant defences in the body (Boligon et al., 2014). The excessive oxidative stress leads to a damage of lipid, proteins and DNA and causes various illnesses including gastric ulcer and delayed wound healing (Handa et al., 2010). Otherwise, the antioxidants provide cell protection through enhancement of the body defence against free radicals, cellular damage and degenerative diseases (Zheng et al., 2014). The action of antioxidants and reduction of oxidative stress play an essential role in the biological therapy of anti-ulcers and wound
healing (Sidahmed et al., 2013). Different approaches (in vitro) are used for the assessment of the antioxidant activities of plants and evaluation of their abilities to inhibit the oxidation reaction caused by free radicals and scavenge these radicals as have been reported by (Awah et al., 2010; Qader et al., 2011). FRAP assay is deliberated to be an accurate method for testing the antioxidants power of therapeutic composites (Henderson et al., 2015). Our data of the ferric reducing antioxidant power (FRAP) assay suggest that C. barometz and V. pubescens leaves extract have higher free radical scavenging than the control antioxidant BHT and revealed powerful antioxidants activities in the reduction of the ferric ion. The previous results agree with others’ report that indicated the medicinal plants containing natural antioxidants contributed to to declining free radicals and deactivate their negative effects (Lai & Lim, 2011). In the scavenging assay of DPPH (2,2-diphenyl-1-pycrilhydrazil) free radical, the proton is abstracted from the antioxidant plants through unstable DPPH radical to have diamagnetic 2,2-diphenyl-1-picrylhydrazyl (Awah et al., 2010). Therefore, the efficiency of any antioxidant plant in DPPH test is dependent on its proton- (hydrogen-) donating capacity. Our results of the DPPH free radical scavenging activity of C. barometz and V. pubescens leaves ethanol extract showed an elevation of the DPPH scavenging when compared to the antioxidant controls: BHT, ascorbic acid, quercetin and gallic acid. These presented outcomes, confirmed other researchers’ reports (Qader et al., 2011) that C. xanthorrhiza and A. paniculata could have a potential DPPH scavenging activity. It is might be due to the resonance effect of the hydroxyl groups attached to the phenolic ring in the plants. This in turn significantly influences the neutralization of free radicals, the reducing of triplet and singlet oxygen, as well as the peroxides’ decomposition (Gwaram et al., 2012).

The antioxidants estimations of phenolic compounds and flavonoids quantities in C. barometz and V. pubescens leaves ethanol extract were total phenolic content (TPC) and
C. barometz and V. pubescens have more TPC values than the antioxidant controls: BHT and quercetin. Additionally, each plant of this study showed TFC values of with equivalent to quercetin. Hence, our observations well agree with the claim of Wu & Yang, (2009); Meena et al. (2011) that many phenolic compounds and flavonoids (The largest group of phenolic compounds) are contained in the genus of Cibotium and Vitex plants and they act as reducing agents in the plants with different bioactivities functions. These contents of plants are considered potent and chelator antioxidants that support particular herb’s medicinal properties (Chavan et al., 2013). In this study, the nitric oxide (NO) and superoxide anion (O₂⁻) radicals scavenging activities of C. barometz and V. pubescens were good with IC₅₀ values based on free radicals scavenging. A similar inhibition of NO and O₂⁻ radicals has been reported by Thatoi et al., (2014) that some medicinal plants have abilities to counteract the free radicals with subsequent decrease in the lipid peroxides. In this regard, C. barometz ethanol leaves extract showed more powerful antioxidant activities than V. pubescens which may suggest that the former contain that might be found more phenolic compounds and flavonoids (Wu & Yang, 2009; Meena et al. 2010).

Thus, the scavenging activities of free radicals have been motivated by consumption of both plants studied and the radicals were deactivated through stabilization and delocalization of the unpaired electrons. Then making the ROS more stable to defend biological molecule from oxidative injuries in the ulcers either gastric or wounds (Roy et al., 2014).

5.1.2 Cytotoxicity

The cytotoxicity study of C. barometz and V. pubescens leaves ethanol extracts and their fractions was assessed for 48 hrs treatments using MTT assay on normal hepatic
human WRL68 cell line to determine any potential cytotoxicity in vitro (Taha et al., 2012). The mitochondrial reduction MTT assay is one of the most frequently used to determine cytotoxicity and cell proliferation.

Our present findings demonstrated that each natural plant and its fractions have ability to proliferate the viability of the WRL68 cell line and did not show any cytotoxic activities against normal liver cells. The similar findings on WRL68 cell line were matched with the earlier studies reported (Ismail et al., 2012; Adjei et al., 2014). Amongst all the fractions, number 1 of *C. barometz* (*CbF1*) and number 2 of *V. pubescens* (*VpF2*) showed the highest viabilities of cells in this test used for further identification procedure. Thus, the cellular proliferative activity of *C. barometz* and *V. pubescens* could be due to the antioxidant and anti-inflammatory activities that lead to offer them as therapeutic alternatives.

**5.1.3 Active compounds of *C. barometz* and *V. pubescens***

The LC-MS results in this current study demonstrate four identified compounds in *C. barometz* leaves (*CbF1*) that are D-glucose (C$_6$H$_{12}$O$_6$), 5-hydroxymethyl-2-furancarboxaldehyde (C$_6$H$_6$O$_3$), alternariol (C$_{14}$H$_{10}$O$_5$), 24-methylenecycloartanol (C$_{31}$H$_{52}$O). These isolated compounds of *C. barometz* leaves are similar to isolated compounds of *C. barometz* rhizome, which has been reported, in the previous study of Wu & Yang, (2009). Phenolic derivatives such as 5-hydroxymethyl-2-furancarboxaldehyde (furan compounds) are found in coffee, prunes, honey, dried food and raisins at the highest levels. It also forms through Maillard reaction, which mostly contribute to desired color, taste and aroma of heated foodstuffs (Capuano & Fogliano, 2011). This might be the cause of using *C. barometz* orally consumed due to with the good and sweet tasty plant and used in some countries of East Asia to treat ulcers. It is considered as a protective factor for cardiovascular co-morbidities, such as obesity,
hypertension, diabetes, and hypercholesterolaemia (Loizzo et al., 2014). Alternariol can be found in sunflower seeds, olives, apples, mandarins and tomatoes that exhibits antioxidant, antifungal and antibacterial and acetylcholinesterase inhibitory activities (Meng et al., 2012; Scott et al., 2012). The inhibition of acetylcholinesterase is necessary to allow a cholinergic neuron to return to its relaxing state after activation and decrease the stress that effective related to gastric ulcer treatment (Jukic et al., 2012). Additionally, 24-methylenecycloartanol that isolated in a previous study from O. europaea (olive) fruits showed antioxidant, antihypertensive, antidiabetic, gastroprotective, anti-inflammatory, neuroprotective activities (Hashmi et al., 2015; Wedler et al., 2015).

On the other hand, the leaves of V. pubescens (VpF2) have five compounds when they were identified by LC-MS. They are caffeic acid (C9H8O4), quercetin (C15H10O7), vitexin (C21H20O10), apigenin (C15H10O5), luteoloside (C21H20O11) similarly with the identified compounds of Vitexagnus castus leaves (Mari et al., 2015). In a previous study of hydroalcoholic extract of Origanum vulgare, the flavonoids namely caffeic acid, quercetin, apigenin, and luteoloside showed a higher efficacy against some species of gram-negative and gram-positive bacteria that lead to be more effective in wound healing with decreasing of inflammation and treat the ulcers (Martins et al., 2014).

Thus, the antioxidant and the anti-inflammatory properties of C. barometz and V. pubescens leaves seem to be related to the phenolic compounds mainly flavonoids (Wu & Yang, 2009; Meena et al. 2010). This pharmacological study confirms the bioactive potential of C. barometz and V. pubescens leaves and provides a reasonable support for its various traditional uses.
5.1.3.1 Gene expression study

Currently, the cellular and molecular mechanisms which critically influence the wound healing quality and how their associated responses can be manipulated using therapeutic intervention, represent key questions in wound therapy either genetic or cellular. Many experimental studies have assisted to elucidate the influences of the bioactive molecular effectors on wound healing and to study the different aspects of oxidative damage attributed to impaired wound healing (Peng et al., 2012). It is reported that various regulations (up or down) of vital genes expressions associated to oxidative destructions inside the cells and also related to assessment of genetic or cellular therapy (Kheirelseid et al., 2010). The pro-apoptotic Bcl-associated X (Bax) and Collagen Type I Alpha 2 (Col1a2) genes are among the altered genes associated with wound repair as previously reported by Xie et al., (2011) and Kandhare et al., (2016).

In our study, the expression comparisons of selected genes; BCL2 associated X protein (Bax) and Collagen Type I Alpha 2 (Col1a2) using real time-polymerase chain reaction (RT-PCR) showed a molecular response of the human skin fibroblast cell line (Hs27) towards the treatments of crude extract of C. barometz leaves (the higher effective antioxidant and anti-inflammatory plant of our study). While the collagen is a principle constituent of the skin tissue regeneration in wounds, Col1a2 gene studies used to evaluate the efficacy of pharmacological inhibitors of fibrogenesis and scarring (Ponticos et al., 2004). Our results of gene expression on Hs27 fibroblast cells demonstrated the apoptotic Bax downregulation and marked Col1a2 expressions in cells treated with the extract of C. barometz leaves. The findings of this study come in concordance with previous research of Kandhare et al. (2016) that showed a positive role of the extract in regulating the expression of regulatory and apoptotic genes to
contribute in accelerating and regulating the wound healing process at the molecular level.

5.1.4 Acute Toxicity

Our findings of acute toxicity test demonstrated that all SD rats treated with *C. barometz* and *V. pubescens* ethanolic extracts have no mortality and toxic signs in the experiment at 2 g/kg and 5 g/kg doses within 14 days after extract administration. The biochemical remarks on liver and kidney of SD rats and their weights were normal appeared in comparison to the control vehicle groups (10% Tween 20) and no significant changes. In addition, they showed no body weight variations or abnormal physiological or behavioural changes at 2 g/kg and 5 g/kg in compared to vehicle group. These outcomes are consistent with the outcomes of earlier studies (Ismail *et al.*, 2012; Hor *et al.*, 2011). Hence, based on our results of the oral administration of each plant in acute toxicity test, it can be concluded that *C. barometz* and *V. pubescens* leaves ethanolic extracts are safe medicinal plants on the SD rats either male or female up to 5 g/kg dose due to the absence of any toxicological implications.

5.1.5 Gastro protective effect study

Several mechanisms are associated with the production of gastric mucosal ulcers. Ethanol directly induces injury to the mucosa of gastric, declining the bicarbonates secretion and the generation of mucus, depressed gastric homogeneity, inhibited prostaglandin synthesis, increased lipid peroxidation, generation of free radicals, and blunted nitric oxide. Ethanol induced ulcer injury to the gastrointestinal mucosa begins with the distraction of the vascular endothelium, consequently increasing the vascular permeability and leading to edema and leucocyte infiltration of the submucosal layer (Al Batran *et al.*, 2013; Golbabapour *et al.*, 2013).
Our findings showed protection of the stomach wall mucosa and reduction of the ulcer area in animals pre-treated with *C. barometz* and *V. pubescens* leaves extracts. Consistently, numerous authors have reported reduction in the ulcer area of the gastric mucosa, increasing the protection of gastric from ulcers in rats (Wasman *et al*., 2011; Sidahmed *et al*., 2013). Ethanol might severely injure the stomach wall mucosa, resulting in elevated neutrophil infiltration into the ulcerated mucosa. Oxygen free radicals originating from penetrated neutrophils in the injured stomach wall impair the outcome of gastric ulcers prevention in rats (Abdulla *et al*., 2010). Neutrophils are a highly important resource of inflammatory mediators and can release powerful reactive oxygen species that are extremely cytotoxic and encourage tissue injury. Additionally, neutrophil accumulation in the stomach mucosa has been shown to provoke microcirculatory abnormalities (Nordin *et al.* 2014). The inhibition of neutrophil permeation throughout inflammation was established to improve gastric ulcer prevention (Al Batran *et al*., 2013).

In this investigation, flattening of the gastric mucosal folds occurred, suggesting that the anti-ulcer outcome of *C. barometz* and *V. pubescens* leaf extract strength is associated with a decline in gastric motility. It has been mentioned in literatures that alteration to gastric motility is involved in the avoidance of tentative gastric injury (Taha *et al*., 2012; Hajrezaie *et al*., 2015). The outcome of our study exhibited intense staining of the glycoprotein secretions of the gastric wall mucosa glands in rats pre-treated with omeprazole or *C. barometz* and *V. pubescens* leaves extracts. Mucus secretion is among the important mechanisms of gastric mucosal defense against necrotizing agents (Salga *et al*., 2012). Mucus and bicarbonate secretion might play a significant role in the ulcer-inhibiting process because the mucus/bicarbonate layer protects newly formed cells from acid and peptic injury (Golbabapour *et al*., 2013).
Oxidative stress may play a major role in the induction and pathogenesis of stomach ulcers, and antioxidant enzymes have been mentioned to play a main defensive role of protection of the stomach wall mucosa against a variety of necrotic agents (Hajrezaie et al., 2012). Antioxidant enzymes could inhibit ethanol-induced gastric damage in rats, and *C. barometz* and *V. pubescens* leaf extract have been demonstrated to contain antioxidants (Meena et al., 2011); thus, it is possible that the gastroprotective properties of *C. barometz* and *V. pubescens* could be due to its antioxidant properties. Antioxidants are responsible for protecting the gastric mucosa from ulceration, as they possess the capability to protect tissue against damages via a radical scavenging mechanism (Sowndhararajan & Kang, 2013). An earlier study provided evidence that ethanol can cause gastric tissue injury through increasing reactive oxygen species (ROS) development (Sidahmed et al., 2013). Consequently, ROS accumulation reduced the GPx activity and increased lipid peroxidation (Liu et al., 2015). GPx can reduce oxidative stress and perform a significant defensive function against ethanol-induced gastric cell damage (Golbabapour et al., 2013). Thus, the detrimental effect of ethanol on the gastric mucosa is clearly linked with decreased GPx activities (Dhiyaaldeen et al., 2014). Moreover, ethanol affects the properties of the gastric tissue by elevating lipid peroxidation that MDA is the major creation of lipid peroxidation. Thus, MDA acts as a marker of ROS-mediated gastric injuries (Sowndhararajan & Kang, 2013). This study indicates that the stomach is protected through pre-treatment with *C. barometz* and *V. pubescens* by increasing the activity of GPx and decrease of MDA level in comparison with ulcer control group.

Our project outcomes indicate that the gastric tissue MDA level was considerably augmented in the ulcer control group, with a significant decrease in the antioxidant enzyme activities of SOD and CAT and of the GSH level in the gastric
homogenate. Pre-treatment with *C. barometz* and *V. pubescens* significantly reduced the malondialdehyde (MDA) concentration level and significantly increased the antioxidant enzyme activities in the stomach homogenates, most likely by inhibiting the production of lipid peroxides from fatty acids in the stomach. In the lipid peroxidation, many free radicals are formed in the body such as reactive oxygen species (ROS) that create a biological destruction in the cells of stomach as result of extreme oxidative stress and decreasing the antioxidant defences in the body (Boligon *et al.*, 2014). The excessive lipid peroxidation leads to gastric ulcer and delayed wound healing (Handa *et al.*, 2010). In addition, the reduction in MDA enzyme by the *C. barometz* and *V. pubescens* extracts in response to the oxidative stress in animals might be resulted of the antioxidant effect of the extract in the stomach homogenate, where the severe damage to the mucous membranes caused by ethanol is prevented. The significantly decreased levels of MDA in animals fed with the plant extract might be due to the decreased oxidative gastric damage (Sowndhararajan & Kang, 2013). The findings are consistent with data published elsewhere (Wasman *et al.*, 2011). Amongst the several pathological trials produced by an inequity among oxidative injury and antioxidant protection systems, lipid peroxidation is a form of oxidative harm that disrupts cell membranes. Similar results have been reported by several researchers (Cuong *et al.*, 2009; Ismail *et al.*, 2012).

The administration of absolute ethanol causes injuries to the epithelial cells, causing a decrease in protein concentrations. SOD and CAT are the main scavenging enzymes that eliminate radicals *in vivo* (AlRashdi *et al.*, 2012). A decline in the activity of these antioxidant enzymes lead the additional accessibility of superoxide radicals, such as superoxide anions and hydrogen peroxide.
Ethanol-generated ROS by unfolding and aggregation of proteins cause damage of proteins. HSP70 is an important endogenous cytoprotective factor. The cells are protected from oxidative stress by HSP70 proteins and allowed refold of the partially denatured proteins. In this study, upsurge of HSP70 could suggest that \textit{C. barometz} and \textit{V. pubescens} protected the stomach through the increase of HSP70 by increasing mucosal blood flow under stress conditions. The induction of HSP70 seems to affect the mucosal protection. In agreement with the results of the current investigation, several studies have reported the upsurge of HSP70 protein to protect the stomach from necrotizing agents. Under stressful and thermal conditions, the HSP70 protein is induced and performs its cytoprotective repair role through its molecular chaperone activity. Ethanol damages the gastric mucosa and produces lesions, and based on our experiments, \textit{C. barometz} and \textit{V. pubescens} treatment in rats exerts its protective role through significant HSP70 induction to reduce lesion development (Meena \textit{et al.}, 2011; Wasman \textit{et al.}, 2011; Zhao \textit{et al.}, 2011).

Following mitochondrial injury and apoptosis activation, Bax, a key pro-apoptotic protein, is translocated to the mitochondria from the cytoplasm (Hajrezaie \textit{et al.}, 2011).

Immunohistochemictric analysis showed that \textit{C. barometz} and \textit{V. pubescens} extracts significantly inhibited increase Bax protein expression. Therefore, these results demonstrate that \textit{C. barometz} and \textit{V. pubescens} extracts exhibits significant protective efficiency against injury in rat’s stomach, which is related to decrease of Bax protein. Numerous studies on this effect have been reported by many investigators (Amaral \textit{et al.}, 2013; Ismail \textit{et al.}, 2012).
5.1.6 Wound healing potential study

The complex natural process of wound healing initiates in response to physical injuries of the skin that restores the function and integrity of damaged tissues. The regenerating in a wounded area involves a set of biochemical events takes place (inflammatory, proliferative, and remodeling phases) with a series of immune mediated cellular and molecular interactions (George et al., 2014). The inflammatory phase includes a well-characterised sequence of immune cell infiltration (Portou et al., 2015), however, the proliferative phase comprises the extensive angiogenesis, collagen deposition, tissue granulation formation, epitheliazation, and wound contraction that result in a smaller amount of apparent scar (Abdulla et al., 2010). Commonly, wound healing can be controlled and accelerated by enhanced scavenging of free radicals as reported previously in the study of Abdulla et al. (2011). The control of chronic wounds such as diabetic wounds is complicated that is a result of delayed the collagen synthesis, impaired endothelial function, impaired epithelialization, defected angiogenesis, and diminished fibroblast proliferation, increased reactive oxygen species, reduce antioxidants and slow wound healing (Chen et al., 2012). Persistent hyperglycemia can enhance the oxidative stress by increasing glucose auto-oxidation and nonenzymatic protein glycation (Simeonova et al., 2016) High levels of blood glucose caused by diabetes can, over time, affect the nerves (neuropathy) and lead to poor blood circulation, making it hard for blood-needed for skin repaor- to reach areas of the body affected by sores or wounds. This can cause them to remain open and unhealed for months, increasing the risk of fungal infections, bacterial infections and gangrene. Keeping blood glucose levels under good control can help to reduce the risk of slow healing wounds now and further in the future. Thus, elimination of reactive oxygen species is an important strategy to improve the healing of wounds in diabetes mellitus patients (Ebaid et al., 2013). The importance of alternative medicine is widely desired for decreasing the risks of delayed wound healing
and reduction of the undesired side effects of drugs. Conversely, the electron donating and free radical scavenging potential of medicinal plants revealed a remarkable bio-efficacy in the treatment of wound ulcers (Zheng et al., 2014). For these and many other reasons, *C. barometz* and *V. pubescens* leaves are being exploited in this work for their bioactivities potential as anti-inflammatory herbs, antioxidants, and wound healing accelerators (Cheng et al., 2010; Mughrabi et al., 2011).

Based on the gross macroscopic appearance, the topical application of *C. barometz* and *V. pubescens* ethanolic extracts on skin excision wound of normal and diabetic rats resulted in improved wound healing rates and a reduction of the healing time of wounds in a dose dependent manner compared with the vehicle (CMC 2%) group. This observation in our work was supported by reduction in the wound area and shortness of the scar on the different days (the 5th and 10th days in normal rats but the 5th, 10th and 15th days in diabetic rats) of dressed wounds with *C. barometz* and *V. pubescens* leaves extracts. The findings of this study come in accordance with the earlier studies of Al-Bayaty et al. (2012) and Kumar et al. (2012) that the natural medicinal plants scavenged the generated ROS and up-regulated collagen production in the treated wound tissues and lead to enhance the wound healing activity. Otherwise, the delayed healing observed in the vehicle groups could be due to the high levels of reactive oxygen species and a higher level of macrophage and neutrophil infiltration. Then followed by the impairment of keratinocytes, endothelial cells, fibroblasts, and collagen metabolism. These outcomes are similar to the outcomes in previous researches (Romero-Cerecero et al., 2014). Moreover, the above results recommended that a high dose (200 mg/mL) of *C. barometz* showed a higher efficacy in accelerating the wound healing process that may be due to their bioactive compounds with their antioxidant and anti-inflammatory properties.
In agreement with Hajiaghaalipour et al. (2013), the histological evaluation of wound tissues using haematoxylin and eosin (H&E) staining in treated groups with each plant extract and Intrasite gel confirmed a demonstration of improved healing observations. Such as decreasing of inflammatory cellular infiltration, increasing of fibroblasts blood delivery to the wound and improved of collagen deposition with a reduced wound scar were appeared in comparison to the vehicle groups in normal and diabetic rats that had a wider wound scar. For the previous reasons and intracellular and extracellular antioxidant activities, the active cellular proliferation enhanced in the wound area then the high wound tensile strength and the accelerated healing of wounds were observed to be similar findings of reported former study (Golbabapour et al., 2013).

Our results of Masson’s trichrome stain of the healed wound tissue in the treated rats with Intrasite gel, C. barometz and V. pubescens leaves showed more collagen deposition with lower accumulative inflammatory cells that proved acceleration of healing activity of wounds with comparing to the samples of vehicle group. Conversely, the stained wound tissue of vehicle group exhibited a less collagen deposition with higher accumulative inflammatory cells. This is found to be likewise with a previous literature of Abdulla et al. (2010) stated that enhancement of collagen in the granulation tissue of wounds is an indicator of increasing the structure skeleton of connective tissues in the skin and the tissue regeneration during the wound repair.

In addition, HSP70 protein has significant functions in the enzymatic folding of proteins and controls the activity of regulatory proteins in wound healing. It highly influences the accurate folding and signal transduction pathways for biochemical enzymes (Rajeshkumar et al., 2013). The reduced expression of HSP70 proteins is usually accompanied with increased of Bax protein expression in various pathological disorders (Al Batran et al., 2013; Halabi et al., 2014). In agreement with these studies, our investigations showed a decrease expression of HSP70 protein and an increase
expression of Bax protein in vehicle groups of healed wound tissue. On the other hand, up regulation of HSP70 protein expression with down regulation of Bax protein expression were seen in the tissue section of treated groups with Intrasit gel and the plants of this study compared to CMC 2% group. Different studies reported observing similar trends (Liu et al., 2015).

During the process of healing in the skin tissue, antioxidant and free radical scavenging activities has a pertinent role in the stages of wound healing. The scavenging enzymes of free radicals are a cytoprotective enzymes that contribute in the reduction and removal of ROS and also regulate the wound healing process (Roy et al., 2014). The superoxide dismutase (SOD) enzyme activity starts when it converts superoxide anions (O$_2^-$) into hydrogen peroxide (H$_2$O$_2$) then produce oxygen and water by catalase (CAT) in lysosomes or by glutathione peroxidase (GPx) in mitochondria (Singh et al., 2014). Our results of the current study demonstrated a marked elevation of SOD activity and in contrary a significant reduction of MDA levels in the homogenates of wound tissue of treated groups of rats with Intrasite gel and C. barometz and V. pubescens leaves in a dose dependent manner compared to the vehicle groups. It could be due to the structural influence of antioxidant and radical scavenging power of the plants in this study that accelerate the wound repair. These findings agree with findings from previous studies (Rajeshkumar & Munuswamy, 2013). In contrary, the homogenates of healed wound in the vehicle groups had a reduction of SOD activities with higher levels of MDA that has been attributed to the rising of ROS production which could decrease the antioxidant enzymes (George et al., 2014).

In this study, C. barometz has higher medicinal properties than V. pubescens possibly due to higher total antioxidants (FRAP assay) that may be lead to increasing of endogenous antioxidants enzymes such as SOD, CAT and GPx in the stomach and skin.
tissues of animals (pretreated and treated) (Singh et al., 2014). The high antioxidant values of *C. barometz* affect positively in animals in preventing and treatment of the ulcers. This is may be due to presence of many phenolic compounds and flavonoids such as caffeic acid, 5-hydroxymethyl-2-furancarboxaldehyde, alternariol and 24-methylene-cycloartanol (Wedler et al., 2015). The antioxidant characteristic of this plant increases the removal of DPPH, NO and superoxide free radicals in the plant and causes decreasing of MDA levels in the animals (George et al., 2014). The high dose of *C. barometz* shows increasing the prevention and treatment of ulcer disease through increasing the Colla2 gene expression and cytoprotective factor HSP70 protein with decreasing of Bax protein expression. Using *C. barometz* leaves in the pretreatment and treatment the SD rats produce an elevation of the mucus content specially glycoproteins (PAS assay) with increasing of PH in the stomach that preserve it from ulceration (Al Batran et al., 2013). It also produced the generation of new capillary vessels below the endothelial cells and accumulation of collagen in the skin for wound healing. In addition, alternariol and 24-methylene-cycloartanol could be regulate insulin secretion and ATP synthesis that help the healing of tissues faster in the wound and prevent the ulcer disease (Chen et al., 2012; Kumar et al., 2012).

Medicinal plants have a wide variety of secondary metabolites such as flavonoids that exhibit antioxidant, anti-inflammatory and antimicrobial properties. This leads to the increase in their usage for treatments of various medical complications and also as dietary supplements (Mancuso, 2015). The presence of these antioxidants and anti-inflammatory properties could be one of the factors that contribute to the wound-healing potential of *C. barometz* and *V. pubescens* extracts and help to avoid the deleterious effects of free radicals.
CHAPTER 6: CONCLUSION

6.1 Conclusion:

In summary, this study demonstrates that *C. barometz* and *V. pubescens* leaves ethanol extract have powerful antioxidants activities, higher free radicals scavenging of DPPH, NO and O⁻ and possess more TPC and TFC values. It shows the safety bioactivity *in vitro* of each plant and their fractions on WRL-68 cell line. On the other hand, the identified of bioactive composites using LC-MS demonstrate four identified compounds in *C. barometz* leaves (CbF1) that are D-glucose, 5-hydroxymethyl-2-furancarboxaldehyde, alternariol and 24-methylene cycloartanol. However, in the leaves of *V. pubescens* (VpF2) have five compounds that are caffeic acid, quercetin, vitexin, apigenin, luteoloside. These compounds have more antioxidants and flavonoids to provide the effectivity of the two plants especially *C. barometz* leaves. Furthermore, this work revealed up regulation of *Colla2* regulatory gene expression and down regulation of *Bax* gene expression on Hs27 fibroblast cells that treated with the crude extract of *C. barometz* leaves (the best effective plant *in vitro*). On basis of *in vitro* study, the outcomes of *in vivo* analyses in acute toxicity test showing that there are no mortality or any sign of toxicological complications even at the high dose of 5 mg/kg. This result suggested the oral administrative safety of the selected plants. Our findings in rats revealed the guarantee antiulcer result of *C. barometz* and *V. pubescens* on gastric mucosal damage induced by ethanol. The two plants of this study arbitrated its antiulcer potential almost certainly throughout its ROS-scavenging activity and defensive result against gastric mucosal damage. From data gained in this experiment, it maybe concluded that gastro protection offered by each plant is mediated through its effect on increasing of mucus production and by its anti-acid secretion. Moreover, the pre-treated of induced ethanol rats with each extract of plants demonstrated decreases of gross
stomach ulcer area and increase of inhibition percentage in a dose dependent manner with respect to the pre-treated rats of ulcer group. In H&E staining, the gastric epithelium of fed rats with each plant resulted in the reduction of the necrotic lesions with visible decrease of haemorrhage, leukocyte infiltration, and oedema-once again indicating to its gastro protective activity. PAS staining intensity of mucosal glycoprotein was amplified because of increasing in the production of mucus that is an indicator of local gastric mucosal defence. Elevations in endogenous enzymes (SOD, GPx and CAT) and decrease in lipid peroxidation (MDA) level, up-regulation of HSP70 and down-regulation of Bax proteins in the pre-treated rats with each plant were established a promising gastro protective agent against gastric ulcer.

Furthermore, the application of C. barometz and V. pubescens ethanolic extracts on the excision’s wound of normal rats resulted in decreasing the wound area and increasing the wound closure percentage depending on the dosage. They had a reduction of the scar width, decline in the numbers of inflammatory cell infiltrates and increased of the fibroblasts, the blood vessels and collagen deposition in the granulation tissues of hematoxylin and eosin (H&E) stained wounds. Moreover, the treated rats with each plant in the study had increasing of collagen accumulations and more generations of new capillary vessels in Masson’s trichrome (MT) staining of the endothelial cells. Up regulated of HSP70 and down regulated of Bax proteins expression, elevated of SOD endogenous enzyme activities and dropped of MDA levels of skin wounds were exhibited in normal and diabetic rats compared to vehicle group.

However, the consequences of diabetic rats that dressed with C. barometz and V. pubescens retain elongated time to be healed more than normal wounds in the rats of experiments. The diabetic wounds in rats, which were dressed with C. barometz and V. pubescens leaves showed decreasing of wound closure area and acceleration of the
healing percentage of wounds in a dose independent manner and time of healing. Moreover, the granulation tissues of wounds using H&E staining exhibited the increases of fibroblast proliferations, vascularization, and collagen depositions with fewer inflammatory cells. Additionally, MT staining of treated rats demonstrated high collagen accumulations and generations of new capillary vessels of the endothelial cells in the dressed rats when compared to carboxymethyl cellulose (CMC 2%) of vehicle group. Conversely, the protein expressions of HSP70 was up regulated and Bax was down regulated of the skin tissue, elevated of SOD endogenous enzyme activities and dropped of MDA levels of diabetic rats were exhibited compared to the vehicle group. Lastly, the enhanced healing of wounds and ulcers in this study could be due to the effects of antioxidant and anti-inflammatory properties of each plant in this study, especially C. barometz.

6.2 Future work:

1) Isolation, purification, and identification of the potential active compounds could be carried out such as 24-Methylenecycloartanol, luteoloside and vitexin that are expected to be responsible for the anti-inflammatory activity.

2) The structures of the compounds can be tested separately using suitable animal models.

3) Additional studies can be performed such as bioavailability, pharmacokinetics and other pharmacological evaluations which are essential to validate the observed properties.

4) The amount of antioxidant should be compared to what actually biologically available in the blood before and after the plant extract administration.

5) Study the mechanism of action for the prevention of oxidative stress by use cell line especially apoptosis and necrosis using flow cytometry method.
REFERENCES


APPENDICES

APPENDIX A

H&E STAINING STEPS

1. The tissues were deparaffinised by subjecting the slides to two changes in xylene, each for 3 min.
2. They were hydrated through sequential rinsing in decreasing grades of alcohol and finally water to eliminate the excess of xylene.
3. The tissue was put in 100% absolute alcohol for 3 min.
4. They put in two weaker solutions (95% and 70% of alcohol for 3 min each).
5. The slides were rinsed with slow-running tap water for 3 min. After that, they were stained with haematoxylin (blue stain) for 10 min, washed with running tap water to remove the excess of blue stain.
6. They were decolorized by sinking them in 0.5% acid alcohol and repeated thrice, followed by washing with tap water for 3 min.
7. Each slide was immersed thrice in 2% sodium acetate (bluing agent), washed with tap water for 3 min to 5 min, rinsed thrice by dipping them in 80% alcohol.
8. They placed in eosin for 5 min, dehydrated by passing through increasing concentrations of alcohol: 95% (5 sec), 95% (2 min), 100% (2 min) and 100% (2 min).
9. Each slide was cleared by passing through three types of xylene for 2 min each. Then each slide was mounted on a glass slide with the mounting neutral medium, Digital Picture Exchange (DPX), and the covers were slipped on carefully to avoid distortion. The different histopathological indices screened were hemorrhage, edema, necrosis, inflammatory and dysplastic changes, erosion, and ulceration.
10. All slides were examined under a light microscope that was connected to special morphometric computer (image analyzer) using Morphology 5.1 Demo Version software.
LIST OF PUBLICATIONS


LIST OF CONFERENCES

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