

**Immunomodulatory, Gastroprotective and Wound Healing
Potential of Malaysian Medicinal Plants (*Phaleria
macrocarpa* and *Tinospora crispa*)**

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**THESIS SUBMITTED
IN FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY**

**FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
Kuala Lumpur
2014**

UNIVERSITI MALAYA

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ABSTRACT

Phaleria macrocarpa (Scheff.) Boerl and *Tinospora crispa* (L.) are considered important traditional medicines commonly used in Malaysia and South East Asian countries. In this study, the ethanol extracts of *P. macrocarpa* fruit and *T. crispa* stems were evaluated for their immunomodulatory, gastroprotective and wound healing potential. In addition, acute toxicity and antioxidant activities were investigated using DPPH and FRAP values as well as the total phenolic and total flavonoid contents. The findings of this study suggest that the high antioxidant activity that was found in *P. macrocarpa* and *T. crispa* was due to the high-level of phenol and flavonoid content. According to the results of the acute toxicity study, *P. macrocarpa* and *T. crispa* should both be assigned to the lowest toxicity class. The potential protective effects of *P. macrocarpa* and *T. crispa* on rat gastric mucosal injury induced by ethanol and to clarify the roles of gastrin, pepsin, prostaglandinE2 (PGE2), superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), and inflammatory mediators; transforming growth factor-beta (TGF- β 1) and tumor necrosis factor alpha (TNF- α). Results showed significant ulcer protective effects through the reduction of the ulcer area by a significant elevation of gastric juice pH and an increase of mucus production, as well as a significant increase in the levels of inflammatory mediators PGE2 and TGF- β 1 and a decrease in TNF- α . In addition, the groups pretreated with *P. macrocarpa* and *T. crispa* exhibited increased significant activity of SOD and CAT but significantly reduced MDA, gastrin and pepsin levels. For wound healing potential, groups were topically treated with the *P. macrocarpa* and *T. crispa* extracts daily for 15 days, and wound healing in this group was significantly accelerated compared with the control group. This result was attributed to a significant increase in TGF- β 1, SOD and CAT, and a significant decrease in the

TNF- α and MDA level in the treated groups. Besides, there was also an effect on the apoptosis process by regulating the expression of BAX and BCL2 genes. To achieve the immunomodulatory effect of *P. macrocarpa*, *T. crispa* and their isolated fractions in activated macrophages to release cytokines as IFN- γ , IL-6 and IL-8, a RAW264.7 macrophage cell line was used to determine immunomodulatory activity by investigating intracellular cytokine expression. The results showed that there is an immunomodulatory effect of *P. macrocarpa* and *T. crispa* and their isolated fractions by increasing RAW 264.7 macrophage cell proliferation in a dose dependent manner, and significant inducing of the intracellular expression of cytokines IFN- γ , IL-6 and IL-8. In conclusion, our results prove that *P. macrocarpa* and *T. crispa* have a significant immunomodulatory effect as immunostimulators, giving protective effects against ethanol-induced gastric ulcers. These extracts also possess wound-healing capability by improving the activity of endogenous antioxidant enzymes and preventing free radical-mediated tissue injury besides significantly stimulating the inflammatory mediators, which also, play an important role in the inflammation process, apoptosis regulation, and the remodeling phase in wound healing.

Abstrak

Phaleria macrocarpa (Scheff.) Boerl dan *Tinospora crispa* (L.) dianggap ubat important tradisional biasa digunakan di Malaysia dan Asia Tenggara negara-negara. Dalam kajian ini, ekstrak etanol *P. macrocarpa* buah dan *T. crispa* stem telah dinilai bagi immunomodulatory mereka, gastroprotective dan luka potensi penyembuhan. Di samping itu, disiasat ketoksikan akut dan aktiviti antioksidan menggunakan DPPH dan nilai FRAP selain jumlah fenolik dan jumlah kandungan flavonoid. Hasil kajian ini dijelaskan aktiviti antioksidan yang tinggi yang ditemui di *P. macrocarpa* dan *T. crispa* milik kandungan peringkat tinggi fenol dan flavonoid. Menurut hasil kajian ketoksikan akut, *P. macrocarpa* dan *T. crispa* menunjukkan bahawa kedua-dua tumbuhan harus diberikan kepada kelas ketoksikan terendah. Kesan perlindungan potensi *P. macrocarpa* dan *T. crispa* pada tikus kecederaan mukosa gastrik disebabkan oleh etanol dan untuk menjelaskan peranan gastrin, pepsin, prostaglandinE2 (PGE2), superoksida dismutase (SOD), katalase (CAT), malondialdehid (MDA), dan pengantara keradangan, mengubahnya pertumbuhan faktor beta (TGF- β 1) dan tumor nekrosis faktor alfa (TNF- α). Keputusan menunjukkan ulser yang ketara kesan perlindungan melalui pengurangan kawasan ulser oleh ketinggian ketara pH jus gastrik, dan peningkatan pengeluaran mukus. Selain itu, peningkatan yang ketara dalam tahap keradangan pengantara PGE2 dan TGF- β 1 dan pengurangan dalam TNF- α . Sebaliknya, kumpulan-kumpulan pretreated dengan *P. macrocarpa* dan *T. crispa* dipamerkan peningkatan aktiviti SOD dan CAT tetapi berkurangan MDA, gastrin dan tahap pepsin. Untuk luka potensi penyembuhan, Kumpulan sedang topically dirawat dengan *P. macrocarpa* dan *T. crispa* ekstrak setiap hari selama 15 hari, dan penyembuhan luka dalam kumpulan ini nyata dipercepatkan berbanding dengan kumpulan kawalan. Keputusan ini attributed untuk meningkatkan

dengan ketara pada TGFβ1, SOD dan CAT, dan penurunan yang ketara dalam TNF-α dan tahap MDA dalam kumpulan yang dirawat. Selain itu, kesan ke atas proses apoptosis yang dengan mengawal ungkapan BAX dan gen BCL2. Untuk mencapai kesan immunomodulatory daripada *P. macrocarpa*, *T. crispa* dan pecahan terpecil mereka dalam macrophage diaktifkan untuk melepaskan sitokin sebagai IFN-γ, IL-6 dan IL-8. Sel macrophage RAW264.7 digunakan dalam kajian kita untuk menentukan aktiviti immunomodulatory dengan menyiasat ungkapan cytokines intrasel. Keputusan kajian kami telah menunjukkan bahawa terdapat kesan immunomodulatory daripada *P. macrocarpa*, *T. crispa* dan pecahan terpecil mereka dengan meningkatkan RAW 264.7 sel macrophage percambahan dos cara bergantung dan penting mendorong ungkapan intraselular cytokines IFN-γ, IL-6 dan IL-8. Kesimpulannya, keputusan kami membuktikan bahawa *P. macrocarpa* dan *T. crispa* mempunyai kesan immunomodulatory sebagai immunostimulator, kesan perlindungan terhadap etanol yang disebabkan ulser gastrik dan mempunyai keupayaan pembedahan lukapenyembuhan yang ketara dengan meningkatkan aktiviti antioksidan endogen enzim dan menghalang kecederaan tisu radikal bebas-pengantara selain itu, ketara merangsang pengantara keradangan. Selain itu, memainkan peranan penting dalam proses keradangan, peraturan apoptosis, dan fasa pembentukan semula dalam penyembuhan luka.

Acknowledgement

First thanks to Allah for help and patience, allowing me for stay far away from my children throughout my period of study. I dedicate this thesis to my beloved mother, father and my boys. In addition, the deepest thanks and gratitude to my mother and father who took care of my children and gave me support. My love and thanks to my children Humam and Ahmed for being patient with me, and my study problems. Thanks to my brothers and sisters for their encouragement.

The greatest thanks and appreciation go to my best friend Assist. Prof. Dr. Ali Ibrahim Ali AL-Ezzy and Iman Fahmi Mustafa who gave me support, stood by my side, and helped me throughout my period of study.

I would like to express my gratitude to my supervisors Prof. Dr. Mahmood Ameen Abdulla and Assoc. Prof. Dr. Salmah Ismail for their guidance and encouragement throughout this work.

I wish to thank the Ministry of Higher Education - Iraq for giving me a chance to study in Malaysia.

I would like to thank University of Malaya, Malaysia for supporting this work through research grant (PV046/2012A).

Finally, I would like to extend my thanks to Malaysia and the Malaysian people for their good dealing, cooperation and for being good hosts to a foreign citizen.

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List of Abbreviations and Symbols

<u>Abbreviation</u>	<u>Description</u>
ADP	Adenosine diphosphate
AKT	Kinase / protein kinase B
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATCC	American type culture collection
BAO	Basal acid output
BAX	BCL-2–associated X
BCL2	B-cell leukemia / lymphoma-2
Bad	BCL-2–associated death protein
Bak	BCL-2 homologous antagonist killer
BHT	Butylated hydroxytoluene
CAT	Catalase
CCK2 receptors	Cholecystokinin 2 receptors
COX	Cyclooxygenase
DMSO	Dimethyl sulfoxide
DMEM medium	Dulbecco's modified eagle medium
DNA	Deoxy ribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
ECL	Enterochromaffin like cell
EGFs	Epidermal growth factors
ELISA	Enzyme-linked immunosorbent assay
FADD	Fas-associated death domain
FAM	6-carboxyfluorescein
FBS	Fetal bovine serum

<u>Abbreviation</u>	<u>Description</u>
FeII-TPTZ	Ferrous tripyridyl triazine
FeIII-TPTZ	Ferritri pyridyl triazine
FGF	Fibroblast growth factor
FGF-2	Fibroblast growth factor-2
FRAP	Ferric reducing antioxidant power
GPx	Glutathione peroxide
HCL	Hydrochloric acid
HeLa	Human cervical carcinoma cell line
HGF	Hepatocyte growth factor
H ₂ O ₂	Hydrogen peroxide
HPRT1	Hypoxanthine phosphoribosyltransferase 1.
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-12	Interleukin 12
IFN- α	Interferon- alpha
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low density lipoprotein
LPO	Lipid peroxidation

<u>Abbreviation</u>	<u>Description</u>
LPS	Lipopolysaccharide
LTs	Leukotrienes
LTC4	Leukotriene C4
LTD4	Leukotriene D4
LTE4	Leukotriene E4
MCF-7	Human mammary cancer cells
MDA	Malondialdehyde
MMPs	Matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF- κ B	Nuclear factor kappa B
NK	Natural killer cell
NO	Nitric oxide
NSAIDs	Nonsteroidal anti-inflammatory drugs
PBS	Phosphate buffer saline
PBMC	Peripheral blood mononuclear cells
PDGF	Platelet-derived growth factor
PGE2	Prostaglandin E2
PGD2	Prostaglandin D2
PGF2 α	Prostaglandin F2 α
PI3	Phosphoinositide-3
PPIs	Proton pump inhibitors

<u>Abbreviation</u>	<u>Description</u>
<i>P</i> value	Level of significance
RAW264.7 cell line	A murine macrophage cell line RAW264.7
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction
S.D	Standard division
SD rats	Sprague Dawley rats
SOD	Superoxide dismutase
SPARC	Secreted protein acidic rich in cysteine
TAMRA	6-carboxy-tetramethyl-rhodamine
TE-2	Esophageal cancer cells
TGF- α	Transforming growth factors α
TGF- β	Transforming growth factor-beta
Th1 cell	T helper cell type1
Th2 cell	T helper cell type2
TIMPs	Inhibitors of metalloproteinase
T _m	Melting temperature
TPTZ	Pyridyl triazine
TRAIL	TNF-related apoptosis-inducing ligand
3T3	Fibroblast cell line

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CHAPTER ONE

1. Introduction

1.1 Gastric ulcer

Gastric ulcer is one of the most common diseases and can be defined as a localized breach of the gastric with tissue destruction at least to the depth of the muscularis mucosa (Tarnawski et al., 2001).

Gastric ulcer is one of the most widely distributed serious and chronic diseases among the world's population. About 10% of the population in the Western world will experience gastric ulcer disease (Barkun & Leontiadis, 2010). In Asia and the South Pacific, gastric ulcer has been diagnosed in 11.5% of the population (Scott et al., 2013). In addition, it is considered a major cause of morbidity and increased health care costs due to rising stress levels that face society, alcohol consumption, nutritional deficiencies and drug usage that in the long term of will cause gastric ulcers as will non-steroidal anti-inflammatory drugs such as aspirin and indomethacin (Abdel-Fattaha & Abdel-Rahman, 2000).

Many medications used to treat gastric ulcer such as antacids, H₂- antagonists and proton pump inhibitor (PPI). H₂- antagonists and proton pump inhibitors such as omeprazole, ranitidine and famotidine work as gastric acid secretion inhibitors. All these drugs have side effects. The main side effects are diarrhea, hypercalcemia (leading to kidney failure), kidney stones, and osteoporosis. Antacids are rarely used for an anti-ulcer evaluation because they are not sufficient for prevention and healing activities (Eid et al., 2010).

The stomach continues to have contact with active harmful agents, and the main and most serious threats to the gastric mucosa as endogenous factors are pepsin together with hydrochloric acid (Vetro et al., 2002). Additional important harmful endogenous factors are the reflux of alkaline duodenal contents containing pancreatic enzymes with bile (Wormsley, 1972). Cigarette smoking and drugs especially steroids, aspirin and NSAIDs have serious exogenous mucosal excitations that can cause mucosal injury (Ko & Cho, 2000; Weil et al., 2000). The efficiency of the stomach to protect itself against these factors is attributed to numerous agents represented in the defense of gastric mucosa. Defense agents such as bicarbonate and mucus excreted through superficial epithelial cells, PGs and gastric mucosal blood flow are considered important in maintaining gastric mucosal safety (Abdel-Salam et al., 2001). Mucus could act as a bacterial trapper agent and be excreted in feces. In addition, mucus has antioxidant activity to reduce mucosal damage caused by factors produced from bacteria and immunocytes (Grisham et al., 1987). Eicosanoid bioactive lipids that include prostaglandins, leukotrienes and thromboxanes play an essential role in gastric physiology. Prostaglandins regularly inhibit gastric acid secretion and preservation of mucosal blood flow (Wallace & Mcknight, 1990). Other factors that have a potential role in maintenance of gastric mucosal integrity are epidermal growth factors and transforming growth factor TGF- β . In the human gastrointestinal tract, TGF- β plays an important function as mediator of the epithelial cell interactions where it regulates epithelial cell proliferation, inflammation and tissue repair. Its expression also increases after acute epithelial injury and in patients with inflammatory bowel disease (Frey & Polk, 2006; Hellmich & Evers, 2006). The body is usually under an active balance between free radical generation and scavenging. The physiological defense systems employed to face free radicals include endogenous enzyme systems, such as superoxide dismutase, catalase, glutathione reduc-

tase and coenzyme Q, as well as exogenous factors like vitamin C, vitamin E, selenium and β -carotene. All these molecules have a central antioxidant function in the fight against oxidative stress through their ability to convert reactive oxygen species (ROS) into stable and harmless compounds or by scavenging ROS with a redox based mechanism (Brambilla et al., 2008). The normalize and scavenge reactive oxygen species level to keep up physiological stability done through enzymatic and non-enzymatic antioxidant protection systems that include catalase (CAT), superoxide dismutase (SOD) and glutathione peroxide (GPx). The existence of an elevated concentration of alteration metal (Fe/Cu) ions, ischemia-reperfusion or drug metabolism causes ROS generation to overpower the cellular antioxidant defense which leads to oxidative stress (Verma et al., 2013).

In recent years, people have tended to use traditional medicine for the treatment of diseases. Medicinal plants are assumed to possess numerous secondary metabolites as flavonoids, alkaloids, terpenoids, tannins and others that help to protect the body from a variety of diseases. Furthermore, there are many medications that have been made from plant sources such as taxol as an anticancer drug from the Yew tree (Slichenmyer & Von Hoff, 1991; Strobel et al., 1996), artemisinin as an antimalarial drug from *Artemisia annua* leaves (Graham et al., 2010; Li & Wu, 2003), and carbenoxolone as an antiulcer drug from *Glycyrrhiza glabra*. Lately, researchers have been showing interest in using natural material from plants to investigate new medications which are more effective and with less side effects to treat gastric ulcer (Borrelli & Izzo, 2000; Farina et al., 1998).

1.2 Wound

Wound is defined as a kind of damage in which skin is torn, cut or ruptured; this is called an open wound. Whereas a closed wound happens when blunt force trauma to the skin results in contusion. The pathological definition of wound refers to an injury that damages the skin's dermis (Boateng et al., 2008). There are many kinds of acute skin wounds, including incision wounds, incomplete thickness damages and wounds including a special tissue lack. Dissimilar wound kinds include diverse phase's process of healing to varying degrees. However, the phases are still the same (Monaco & Lawrence, 2003). The healing of wounds comprises a series of events. These events occur in an accurate to regulate mode. There is overlapping in the wound phases, but they are described in a linear manner to make it clear. The phases of wound healing include hemostasis, inflammation, cellular migration and proliferation, protein synthesis and wound contraction, and finally the remodeling phase. In the healing of the wound, activation of complicated net cells of blood, tissues, growth factors and cytokines cause increased cellular activity that causes rising metabolic requests for nutrients (David & Heather, 2000).

Deficiencies in nutrition could disrupt healing of the wound. Many nutrition agents which might enhance healing wound time and results are needed to repair the wound. Epithelial and bone formation require vitamin A, important for immune function. Vitamin C is essential for collagen creation, as a tissue antioxidant and for good functioning of the immune system. The main lipid soluble antioxidant in the skin is vitamin E (Mackay & Miller, 2003). Excess ROS result in the killing of fibroblasts, and skin lipids will become less flexible. Because of this, antioxidants seems to be significant in the effective treatment and management of wounds. Antioxidants diminish these adverse effects of wounds through eliminating products of inflammation (Houghton et al.,

2005). A possible mechanism for antioxidant defense is the direct interface of the extracts or compounds and hydrogen peroxide instead of changing the cell membranes and controlling damage. These compounds have a vast radical scavenging capacity and have been shown to enhance wound healing. Because of this researchers have tried to find medication from natural sources to cure wounds (Ipek et al., 2012). Numerous cytokines responsible for the stimulation of fibroblast and the synthesis of collagen include TGF- β , EGF and PDGF. Various factors related to the patient as well as to the wound influence the rate of collagen synthesis, and this includes age, tension, pressure, and stress (Velnar et al., 2009). Probably TGF- β and cytokines participate in contraction of the wound (Huebener & Schwabe, 2012). TGF- β , TNF- α , vascular endothelial growth factor and endothelin-1, are all important enhancers of cell proliferation, migration, chemotaxis and angiogenesis in the wound healing process (Tandara & Mustoe, 2004).

In the wound healing process, some probable signals for the expulsion of cells involved in tissue repair have been proposed, but the mechanisms of how the cells expire require to be studied. Cells participate in each phase, expiring through one of three probable ways, which are apoptosis, necrosis and emigration. Eighteen member proteins of the BCL2 family have been discovered to be influencers of the apoptosis pathway, and the BCL2 family proteins either promote or prohibit apoptosis. The pro-apoptotic proteins involve BAX, Bad and Bak, whereas BCL2 and BCL-xL are anti-apoptotic proteins. Apoptosis plays a critical function in the synchronization of speedily altering cell residents, which is complicated in all tissue healing. For a massive common wound, controls of proliferation cause an acceleration of the wound's closure. Sometimes the equilibrium between the increase and decrease in number of cells forfeits its equilibrium and causes pathologic tissue repair (Rai et al., 2005).

1.3 Immunomodulatory

The immune system is a remarkably developed defense system found inside vertebrates to guard them from attacking factors of illness. It is capable of producing a diverse range of molecules and cells which are able to distinguish and reduce the unlimited changes of external and unwanted agents. The modulation of the immune system refers to some alteration in the immune response, which could include stimulation, amplification and expression or inactivation of the immune response. Therefore, the immunomodulator is a substance used for its effect on the immune system. There are commonly two kinds of immunomodulators based on their effects, immunostimulators and immunosuppressors. They have the ability to mount an immune response or defence against pathogens or tumors (Saroj et al., 2012). Material which has shown a modifying immune system response to a threat, is an immunomodulator. Immunomodulators modulate and potentiate the arm of the immune system by keeping it in a highly prepared state against threat (Mishra et al., 2008).

In different parts of the world, plant extracts have been widely investigated for the possibility of immunomodulation. Many of the studies have demonstrated the isolation of potential bioactive molecules (Alamgir & Uddin, 2010). For example, *Acorus calamus* rhizome extract inhibited the growth of several cell lines for humans and mice. It also inhibited the production of nitric oxide (NO), interleukin-2 (IL-2) and tumor necrosis factor- α (TNF- α). In addition, it can cause down-regulation expression of the CD25 marker (Mehrotra et al., 2003) and many plant derived compounds like sterols, sterolins, polysaccharides, alkaloids, flavonoids, lectin and glycoprotein are used as immunomodulators (Kolm et al., 2007; Lien et al., 2003).

Modulation of the immune response to reduce diseases has long been an attraction for researchers. There have been many studies lately on ethnomedicinal plants as immunomodulatory agents. Immunopharmacology is a relatively new division of pharmacology, which aims to search for immunomodulators. The possible usage of immunomodulators for clinical medicine can comprise reconstruction for immune deficiency, for example, treatment of the suppression of normal or exaggerated immune roles like treatment of autoimmune diseases and AIDS. An important source of immunomodulators is medicinal plants and their active components. Therefore, the improvement of drugs from natural compounds for their immunomodulation and anti-tumor potential is an interesting project (Alamgir & Uddin, 2010).

1.4 Medicinal plants

In the past, plants supplied humans with sources of dye, food, perfume, gum, resin, fiber and many other useful products. Nowadays, ethno-pharmacologists are paying more attention to investigating the bioactive properties and phytochemical analysis of medicinal plants to treat a variety of diseases. Medicinal plants have a main therapeutic function and could be used as a natural medicine source for treating different diseases (Gupta & Sharma, 2006). Recently, many researchers endorsed the traditional belief in utilizing medicinal plants to treat a variety of diseases involving gastric ulcer. Many studies have reported that plants possess various bioactivities (Alama et al., 2009; Yadav et al., 2011). *P. macrocarpa* and *T. crispa* are traditional drugs that are used in the treatment of inflammation, cancer, and diabetes (Hendra et al., 2011a; Mardina et al., 2011). In this study, we used *P. macrocarpa* fruit and *T. crispa* stem extracts to investigate their immunomodulatory, gastro protective potential and their ability in wound healing. Ethopharmacological evidence indicates the utilization of *P. macrocarpa* and *T. crispa* by humans for several purposes. The chief purpose of the plants was as a basis

for food additives. However, the plants are also used as a source of natural medicine agents and have an extensive diversity of secondary metabolic materials such as alkaloids, tannins, terpenoids and flavonoids. There have been studies *in vitro* of their antioxidant, anti-inflammatory properties and this has been used for developing drugs or dietary supplements (Mohammed et al., 2012; Yosie et al., 2011).

Traditionally, *P. macrocarpa* and *T. crispa* have been used to in the treatment of hemorrhoids, cancers, diabetes, blood diseases, allergies, acne, migraine and various skin diseases (Noipha et al., 2010; Widyasari et al., 2011). Previous studies reported isolating many active compounds from *P. macrocarpa* and *Tinospora crispa* like phalerin, mangiferin, benzophenone glucopyranosides, mahkocide, kaempferol-3-O-b-D-glucoside, dodecanoic acid, and palmitic acid from various parts of *P. macrocarpa* (Asih Triastuti & Choi, 2008; Tandrasasmita et al., 2010), tyramine and epinephrine from the fraction of *T. crispa* extract, which are reported to act in the control of blood pressure (Pramana et al., 2011) and tinosporaside, cordioside, and columbin (Ahmed et al., 2006).

1.5 The objectives of this study

As an expansion of our initial study on the biological screening of local medicinal plants and gastroprotective, wound healing and immunomodulatory, we identified *P. macrocarpa* and *T. crispa* as potential candidates for further investigation. The present study was conceptualized with the following:

1.5.1 General objectives

To investigate gastroprotective, wound healing and immunomodulatory potential for *P. macrocarpa* and *T. crispa* ethanol extracts as well as to identify the active constituents of the both plants.

1.5.2 Specific objectives

1. To investigate the antioxidant activity (DPPH and FRAP methods), total phenolic and total flavonoid contents *in vitro*.
2. To investigate the acute toxicity of *P. macrocarpa* and *T. crispa* extract in rats.
3. To evaluate the gastroprotective ability of *P. macrocarpa* and *T. crispa* extract by inducing gastric ulcers in rats.
4. To evaluate the wound healing potential of *P. macrocarpa* and *T. crispa* by inducing excision wounds in rats.
5. To estimate the change in the gene expression of apoptosis markers (BAX and BCL2) in the wound healing model.
6. To investigate the immunomodulatory effect of the both plants *in vitro* using a RAW264.7 cell line.
7. To fractionate and identify the active constituents of both plants with immunomodulatory effect *in vitro*.

CHAPTER TWO

2 Literature review

2.1 Gastric ulcer

2.1.1 Definition

Gastric ulcer is one of the most common diseases. Gastric ulcer can be defined as a localized breach of the gastric with tissue destruction at least to the depth of the muscularis mucosa Figure 2.1 (Tarnawski et al., 2001). Gastric ulcer is one of the most widely distributed chronic diseases amongst the world's population and around 10% of the population in the Western world will experience gastric ulcer disease (Barkun & Leontiadis, 2010). In Asia and the South Pacific, gastric ulcer has been diagnosed in 11.5% of the population (Scott et al., 2013). In a previous study which was done in Malaysia and consisted of 124 participants diagnosed as having gastric ulcer, gastric erosions or duodenal ulcer. Based on ethnicity, Malays comprised 69.4% of the sample, whereas 24.2% were of Chinese, 2.4% of Indian, 2.4% of Kampuchean and 1.6 % of Thai ethnic origin. This study was conducted in the northeastern peninsular Malaysia, where Malays are the overwhelming majority, constituting more than 98% of the population, so they comprised the major percentage in the study (Raj et al., 2001).

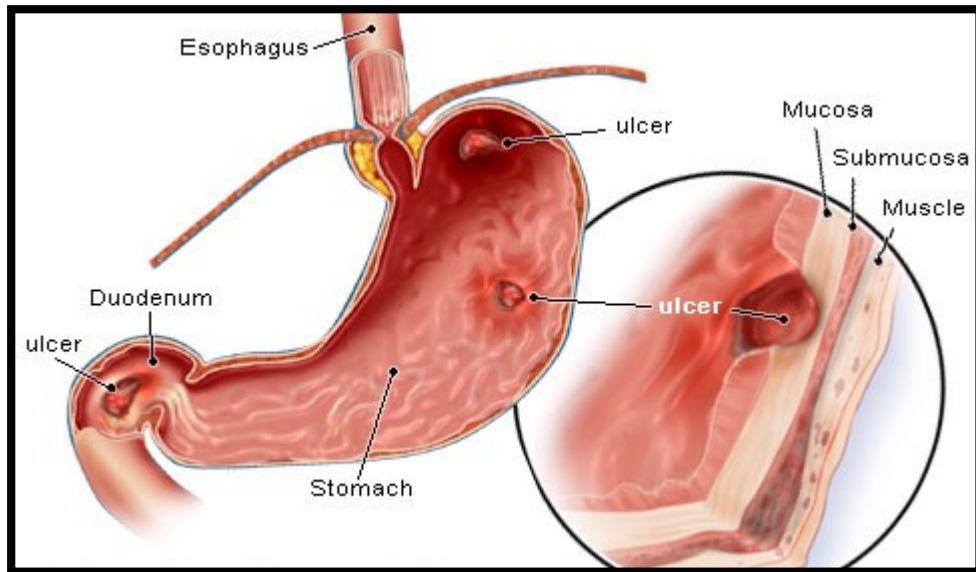


Figure 2.1: Gastric ulcer disease. Adapted from: (Marks, 2012).

2.1.2 Possible etiology and risk factors of gastric ulcer

2.1.2.1 Acid output

In up to one third of patients with gastric ulcer, maximal acid output and basal acid output (BAO) are raised. The rise in BAO might reveal that a significant percentage of patients have gastric ulcer. In addition, an increase in gastric acidity is also observed in some patients with gastric ulcer. Many of these ulcers are situated at the end portion of the stomach, where, in one study, 955 of all gastric ulcers were found (Aihara et al., 2003).

2.1.2.2 Nonsteroidal anti-inflammatory drugs (NSAIDs)

NSAIDs drugs are known to be related to gastrointestinal poisonousness and also to the creation of gastric and duodenal ulcers. As many as 4 to 10 % of patients who take a daily therapeutic dose of NSADs develop gastric ulcer after three months of starting therapy and up to one percent of those gastric ulcers are clinically significant. Factors related to the rising risk of gastric ulcer found in NSAIDs usage are being female,

and/or elderly and heavy and long-term use of NSAIDs drugs (Chan & Leung, 2002; Laine et al., 2008).

2.1.2.3 Life style factors

Lifestyle factors play a role as risk factors that can elevate the possibility of having gastric ulcer disease, such as smoking, which might accelerate gastric emptying and decrease the pancreatic bicarbonate formation that may cause gastric ulcer. Alcohol use causes gastric mucosal irritation and nonspecific gastritis and also gastric reflux, one of the chief causes of gastric ulcer (Chisholm, 1998).

2.1.2.4 Genetics

More than of 20% of patients possess a family history of gastric ulcer in association with (5-10) percentage of the control group. The reason for this genetic relation is not clear. A rare genetic relation has been found between familial hyperactive pepsinogenemia type I (genetic phenotype causing improved secretion of pepsin) gastric ulcers. Related with gastric acid with gastric acid hyper secretion and the progress of gastric ulcer (Atherton, 2006).

2.1.2.5 *Helicobacter pylori* infection

H. pylori bacteria are small, microaerophilic, spiral in shape, gram-negative rods. The existence of *H. pylori* in the stomach and duodenum may be the most common bacterial infection in the world (Atherton et al., 1995). *H. pylori* infection is usually observed as the most important etiologic agent in the progress of gastroduodenal pathology (Chan & Leung, 2002).

2.1.3 Drugs used to treat gastric ulcer

There are several drugs used for treating gastric ulcer. These medications have an effect on the specific receptors and activate certain mechanisms, which contribute to gastric ulcer therapy by neutralizing gastric acid secretion, scavenging free radical mediated by ulcerogenic agents or by promoting mucosal defenses. Antacids such as maalox and mylanta are the first optional drugs used to mitigate heartburn and dyspepsia. Unfortunately, this group is inefficient in preventing or curing gastric ulcers, but it can be useful via these two mechanisms: neutralizing acidity inside the lumen of stomach with the combination of three basic compounds of calcium, magnesium and aluminum or they may guard the mucosal layer of stomach through raising bicarbonate and mucus secretions (Chisholm, 1998).

Antibiotics are another group that can be used to treat gastric ulcers caused by bacterial infection *H. pylori* (Hentschel et al., 1993). Amoxicillin is an effective antibiotic used to diminish bacterial growth and it has been widely used as a positive reference drug to evaluate antibacterial activity particularly *H. pylori* (Malfertheiner et al., 2007).

H2 blockers are a group of drugs, including cimetidine and ranitidine, that are used as a standard treatment for gastric ulcers before proton pump inhibitors and antibiotic treatments against *H. pylori* were developed. They act as suppressors of acid production by blocking histamine, a cytokine molecule created through the body that promotes acid secretion in the stomach. Although there are many researchers using H2 blockers cimetidine and ranitidine (Chisholm, 1998) as the standard group for antiulcer evaluation experiments, these drugs cannot cure ulcers and they are effective only for duodenal ulcers (Wallace, 2005).

Proton pump inhibitors (PPIs) are the common choice of drug to be administrated to patients with gastric ulcers. The mechanism of action is via blocking the stomach acid

secretion by suppressing H^+ / K^+ ATPase. This enzyme is responsible for regulating acid secretions present in the parietal cells of stomach glands. Existing PPIs that are accepted for the prevention of ulcer and for healing comprise omeprazole (Prilosec OTC), esomeprazole (nexium), lansoprazole (prevacid) and rabeprazole (aciphex) (Wallace, 2005). All these drugs have lateral effects, the main lateral effects being diarrhea, hypercalcemia (leads to kidney failure), kidney stones, and osteoporosis. Antacids are rarely used for anti-ulcer evaluation because they are not sufficient for prevention and healing activities (Eid et al., 2010). In this project, omeprazole was used as the positive control group as it has widely been used as a standard drug to evaluate anti-ulcerogenic activity in a variety of plant extracts. Several studies have used omeprazole as a positive control drug against the ethanol induction model based on the proposal mechanism that omeprazole is able to enhance cytoprotection and scavenge oxidative stress arbitrated by ethanol-induced ulcers (Alirashdi et al., 2012; Gupta et al., 2005).

2.1.4 Gastroprotective factors and gastric mucosal integrity

The stomach is always in continued contact with potentially harmful factors. Threats to the gastric mucosa as endogenous factors are pepsin together with hydrochloric acid (Vetro et al., 2002). Additional harmful endogenous factors are the reflux of alkaline duodenal contents having pancreatic enzymes with bile (Wormsley, 1972). Cigarette smoking, drugs especially steroids, aspirin and NSAIDs are serious exogenous mucosal excitations that can cause mucosal injury (Ko & Cho, 2000; Weil et al., 2000). The efficiency of the stomach to protect itself against these factors has been attributed to numerous agents represented in the gastric mucosal defense. These defense agents involve mucus and bicarbonate excreted by surface epithelial cells, sulfhydryl compounds, prostaglandins and gastric mucosal blood flow, and are considered important in maintaining gastric mucosal safety (Abdel-Salam et al., 2001).

The barrier that was formed between the body and the luminal environment is the gastrointestinal mucosa, that not only has nutrients but is loaded with actively hostile microorganisms and toxins. The gastrointestinal tract barrier is composed of two major parts; the first which is the epithelial cell lining (the digestive tube) and the second which are secretions that influence the epithelial cells and preserve their barrier function. What is crucial for barrier integrity is the maintenance of intact epithelium and the balance between cell proliferation and cell death (Gary & Wallace, 2006). The gastric mucosa is consistently exposed to a mix of endogenous and exogenous injuring agents including gastric acid, bile, drugs and microorganisms. The agents responsible in mucosal protection are; mucus secretion, mucosal microcirculation, acid inhibition, prostaglandins and inflammatory mediators (Abdel-Salam et al., 2001).

Mucus completely coats the gastrointestinal epithelium that is formed by cells. Mucus has a critical function in justifying shear pressures on the epithelium and shares to barrier function. Carbohydrates on mucin molecules react with bacteria, which helps in avoiding colonization by causing acceleration, aggregation and clearance (Adrian & Flemström, 2005).

Mucus can act as a bacterial trapper agent and is excreted in feces. In addition, mucus has an antioxidant activity that reduces mucosal damage caused by factors produced by bacteria and immunocytes (Grisham et al., 1987). Mucus has an important constitutional role in the mucosal surface, which assists in the maintenance of a pH at the surface that is near neutral as well as in performing as a physical barrier against luminal pepsin (Allen et al., 1993).

Eicosanoids are bioactive lipids that include prostaglandins, leukotrienes and thromboxanes that play an essential role in gastric physiology; prostaglandins regularly inhibit gastric acid secretion (Callison et al., 1976; Dey et al., 2006). Previous studies have

proved the inhibitory effect of PGE₂ on acid output in mice, rats, dogs, monkeys and humans (Main & Whittle, 1973; Newman et al., 1975; Robert et al., 1967). Exogenous or endogenous prostaglandins stimulates most of the mucosal defense mechanisms (Wallace & Granger, 1996) and continued output of PGE₂ and prostacyclin are necessary for the safety of the gastric mucosa (Wilson, 1990). Prostaglandins have a significant role in the preservation of mucosal blood flow (Wallace & Mcknight, 1990), as well as encouraging bicarbonate and mucus exudation in the stomach (Allen & Garner, 1980).

Luminal PGE₂ production increased seven fold over basal values after exposing the stomach to 150 mM HCl at pH 0.8 for 5 min in humans (Aly et al., 1985). Prostaglandins preserve gastric mucosal reliability through stimulating mucus and bicarbonate excretion, preventing acid secretion, mast cell initiation and apoptosis, lessening leukocyte devotion to the endothelium, accumulative or preserving mucosal blood flow and therefore avoiding ischemia (Atay et al., 2000).

Szabo et al. (1985) showed that vascular injury donates to the development of hemorrhagic erosions in rats after intragastric administration of ethanol using vascular tracers. Intragastric administration of absolute ethanol stimulated increased vascular permeability within 1-3min after one hour of 100% ethanol exposure. The areal density of grossly visible hemorrhagic lesions also increased patently. Conspicuous hemorrhagic damage to the glands of the gastric mucosa occurred due to obvious lesions in the blood vessels and increased vascular permeability, so vascular injury could be considered early evidence of pathogenic factors in the expansion of ethanol induced gastric hemorrhage corrosion. In addition, it was found that pretreatment with prostaglandin F₂beta reduced ethanol injury. In previous studies, Tarnawski et al. proved that deep necrosis lesions in the gastric mucus layer from absolute ethanol can be totally prevented by 16,16-

dimethyl PGE₂ (Tarnawski et al., 1985). Robert et al. showed that pretreatment with 16,16-dimethyl PGE₂ (10g/kg in 1ml) prevented ethanol from causing histologic alterations (Robert et al., 1992). Severe mucosal damage, extending to the capillaries of the gastric glandular mucosa, that was induced in rats by absolute ethanol for 1 min, can be reduced by pretreatment with PGF at a dose of 0.5mg/100g (Trier et al., 1987). A large number of endocrine and paracrine agents affect the normal proliferation of gastric epithelial cells and propagation to reaction as the wound causes ulceration. Numerous enteric hormones improve the proportion of propagation. Prostaglandins, especially PGE₂ and prostacyclin, are known to have a cytoprotective effect on the gastrointestinal epithelium. The cytoprotective influence seems to result from a complex ability to improve mucosal bicarbonate and mucus secretion to rising mucosal blood flow. Other factors that have a potential function in the preservation of gastric mucosal safety are the epidermal growth factor, transforming growth factor-beta (TGF- β), fibroblast growth factor and hepatocyte growth factor. These peptides impasse a corporate receptor and improve epithelial cell proliferation, and additional molecules have a critical role for mucosal safety and barrier job as nitric oxide (Barnard & Mchugh, 2006). The pathways of the TGF- β superfamily signal are simple controllers of the cellular routes comprising differentiation, proliferation, migration, survival and physiological processes like embryonic development, angiogenesis and wound healing. Variations in these pathways are caused by either somatic mutations or germ-lines, and changes in expression of these signaling pathways frequently result in human disease (Gordon & Blobel, 2008). In the human gastrointestinal tract, TGF- β plays a crucial role as a mediator of epithelial cell interactions where it regulates epithelial cell proliferation, inflammation and tissue repair; its expression also increases after acute epithelial injury and in patients with inflammatory bowel disease. TGF- β null mice died 20 days after birth due to

catching a multifocal inflammatory disease in the stomach and intestine (Frey & Polk, 2006; Hellmich & Evers, 2006). The gastric mucosa is constantly open to actively injurious factors and the protection of mucosal integrity is secured through a difficult structure of interacted mediators. Exaggerated ethanol administration causes gastritis categorized by mucosal edema, cellular exfoliation, sub-epithelial hemorrhages, cell infiltration, inflammation and ulcers. Exogenous compounds, mostly acetylsalicylic acid, NSAIDs drugs and too much ethanol result in corrosive lesions in the gastrointestinal mucosa (Jahovic et al., 2007). Ethanol causes increasing superoxide anion activity, lipid peroxidation and hydroxyl radical production in the gastric mucosa (Bagchi et al., 1998). DNA damage, oxidative stress, increased xanthine oxidase activity, malondialdehyde level and decreased total glutathione content in gastric mucosal cells are prompted by acute ethanol (Marotta et al., 1999). Oxidative stress that disturbs the energy metabolism of mitochondria and has a serious function for pathogenic ethanol-induced gastric mucosa injury is another hypothesis suggested explaining ethanol-induced gastric mucosa injury (Pan et al., 2008). The body is usually balanced between free radical generation and scavenging. The physiological defense systems which face free radicals include the endogenous enzyme systems, such as superoxide dismutase, catalase, glutathione reductase and coenzyme Q, as well as exogenous factors like vitamin C, vitamin E, selenium and β -carotene. All these molecules have an important antioxidant function in the fight against oxidative stress through their ability to convert reactive oxygen species (ROS) into stable and harmless compounds or by scavenging ROS with a redox-based mechanism (Brambilla et al., 2008). Physiological replies to stressors cause increased activity of the hypothalamic pituitary adrenal axis and alterations in the gastrointestinal tissue. This rise in adrenocortical action is related to the rise in the incidence of gastric ulceration. Oxidative stress in the stomach is the cause of

stress ulcers and leads to gastric ulcers (Suzuki et al., 2012). The activation of additional ROS production or a decrease in antioxidant defenses are caused by oxidative stress which is a case of boosted levels of ROS. The pathogenesis of gastric inflammation, ulcerogenesis, *H. pylori* infection, life style associated diseases such as hypertension, atherosclerosis, ischemic heart diseases, diabetes mellitus and malignancies have all contributed to oxidative stress (Suzuki et al., 2011). The influence of ethanol on the gastric mucosa is multifaceted and complicated. It might be associated with a disruption of balance between gastric mucosal protection and offensive agents. Gastroprotective factors preserve the safety of the gastric mucous layer, microcirculatory system, bicarbonate, epidermal growth factor, prostaglandins and epithelial cell recovery, whereas the gastric mucosa is naked to gastric acid and pepsin. Damage to the vascular endothelial cells of the gastric mucosa prompts microcirculatory disturbance, and hypoxia linked to the overproduction of oxygen radicals is caused by ethanol (Suzuki et al., 2012). The generation of ROS is usually a waterfall of responses, which begins with the creation of superoxide that quickly dismutates to hydrogen peroxide. Direct or indirect injurious agents may be caused by free radicals through their reaction with other components in the cell. Enzymatic and non-enzymatic antioxidant protection systems that include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxide (GPx) keep up physiological homeostasis through scavenging and regulating overall ROS levels. The existence of raised alteration metal (Fe/Cu) ions, ischaemia-reperfusion or medication metabolism causes generation of ROS to overpower the cellular antioxidant defense which leads to oxidative stress (Verma et al., 2013).

2.2 Wound

2.2.1 Definition

A wound is defined as any kind of damage where the skin is torn, cut or ruptured; this is called an open wound. Whereas a closed wound happens when blunt force trauma to the skin results in contusion. The pathological definition of a wound is a sharp injury that damages the skin's dermis (Boateng et al., 2008). There are many kinds of acute skin wound, including incision wound, incomplete thickness damage and wound including special tissue lack. Dissimilar wounds kinds include diverse phase's process of healing to a varying degree. However, the phases are still themselves the same (Monaco & Lawrence, 2003). The healing of a wound comprises a series of events. These events occur in an accurate to regulate mode. There is overlapping in the wound phases, but they are described in a linear manner to make it clear. The phases of wound healing includes hemostasis, inflammation, cellular migration and proliferation, protein synthesis and wound contraction and finally the remodel phase (David & Heather, 2000). The healing of a wound contributes to the activation of complicated net cells of blood, tissues, growth factors and cytokines which cause increasing cellular activity that causes a rising metabolic request for nutrients. Deficiencies in nutrition could disrupt the healing of wound. Many nutrition agents are needed for wound repair which might enhance healing wound time and results. Epithelial and bone formation require vitamin A, which is important for immune function, and vitamin C which is essential for collagen creation, as a tissue antioxidant and for the function of the immune system. The main lipid soluble antioxidant in the skin is vitamin E (Mackay & Miller, 2003). Because of this, researchers are trying to find medication from natural sources to cure wounds.

2.2.2 Phases of wound healing

Wound healing follows a foreseeable sequence of events that occurs in an accurately constructed way. The phases of wound healing overlap and can be described in a linear manner for the intention of clarification. Wound healing is characterized by five phases: hemostasis, inflammation, migration of cells and proliferation, synthesis of protein and contraction of wound and the remodeling phase (Figure 2.2).

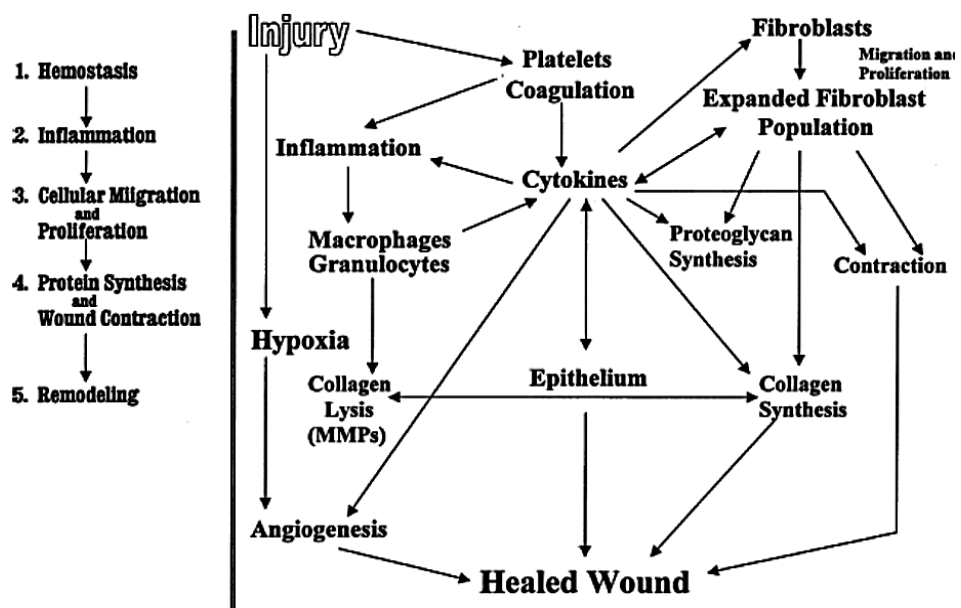


Figure 2.2: The cascade of acute wound healing. The propulsion of acute wound healing from hemostasis to the final phases of remodeling is dependent on a complex interaction of varied acute wound-healing events. Cytokines play a fundamental role in wound healing and act as a central signal for various cell types and healing events. Cited by (Monaco & Lawrence, 2003).

2.2.2.1 Hemostasis

All hazardous traumas generate vascular damage and therefore start the molecular and cellular response which promotes hemostasis. Accomplishment of hemostasis is vital for the healing process. Vasoconstriction, platelet aggregation and fibrin deposition are primary contributors of hemostasis which results from the coagulation cascades. Clot formation is the outcome of hemostasis. The primary composition of clots is a fibrin net with gathered platelets associated with surrounding cells (Lawrence, 1998). The clot formation consequence is ingrained. The external environment causes loss of additional fluid and electrolytes from the wound site and the delimitation of contaminants. Fibrin is the net substance to the contingent wound medium on to the fibroblasts and other cells migration to begin the healing process. There are three stages in the hemostasis phase:

2.2.2.1.1 Vasoconstriction

The release of vasoactive amines contributes to the initiation of vasoconstriction which happens when the dermis is entered. While stimulus of the sympathetic nervous system liberates epinephrine into the peripheral circulation this causes local norepinephrine release. Further vasoconstriction is also attributed to prostaglandins such as thromboxane that are secreted from the injured cells (Monaco & Lawrence, 2003).

2.2.2.1.2 Platelet aggregation

Damaged cells caused releasing to tissue factors that exposure to platelet aggregation and fibrinogen. Liberate the substances of alpha granules, dense bodies and lysosomes they happen after aggregation and adhesion of platelets inside their cytoplasm (Cines et al., 1998). A variety of proteinaceous and immunomodulatory agents, which participate in the primary and late phases of healing, can be found inside the alpha granules which are mainly, adhesive proteins (vonwillebrand factor VIII, fibrinogen, fibronectin and

thrombospondin) as well as,, platelet derived growth factor (PDGF), fibroblast growth factor-2 (FGF-2), transforming growth factors α and β (TGF- α & TGF- β), platelet derived epidermal growth factors (EGFs) and endothelial cell growth factors. The TGF- β , PDGF and FGF-2 are the greatest substantial. Calcium, serotonin, ADP and ATP are the necessary fuel providing compounds that participate in the process of healing found inside dense bodies. These compounds share in prompting the coagulation cascades (Griendling et al., 2000).

2.2.2.1.3 Fibrin and the coagulation cascades

The coagulation waterfall is a compound of intrinsic and extrinsic ingredients which are triggered separately. The extrinsic cascade is essential whereas the intrinsic cascade is not involved in the normal healing process. Thrombin is produced through the activation of the intrinsic and extrinsic pathways that catalyze the alteration of fibrinogen to fibrin. Vascular permeability and facilitation of the extravascular migration of inflammatory cells are increased by thrombin stimulates. Fibrin consists of the net used for steadying of the platelet plugs and is a vital element for the temporary matrix that changes rapidly in the wound after damage. Serum and platelets derived from vitronectin coats the fibrin and this accelerates the binding of fibronectin that forms via fibroblasts and epithelial cells (Dahlbäck, 2005).

2.2.2.2 Inflammation

Erythema, edema, heat, and pain are characteristic features of inflammation. The increase in vascular permeability and the consecutive movement of leukocytes into the extravascular area grant a special privilege of inflammation at the tissue level (Figure 2.3). Conducting inflammation cells to the damaged region to break down bacteria and also to remove wreckage from dead cells and the broken matrix is one of the fundamental functions of inflammation so that the repair processes can proceed (Li et al., 2007).

Erythema and heat, as signs of inflammation, develop soon after injury due to vasodilatation. Vasodilatation begins after the start of vasoconstriction, which inverts 10-15 min next wound. To allow the infiltration of plasma from the intravascular area to the extravascular chamber, the endothelial cells lining for capillaries in the closed wound form a gaps between them (Monaco & Lawrence, 2003). The pain sensation, which is a cardinal sign of inflammation, comes from the accumulation of fluid into the area that generates edema. Numerous agents mediate the switching from vasoconstriction to vasodilation. Endothelial products and mast cell derivative factors such as leukotrienes are a group of arachidonic acid metabolites referred to as LTs. Leukotriene B₄ is a potent chemotactic factor that induces aggregation of neutrophils, whereas LT₄, LTD₄ and LTE₄ cause vasoconstriction and increased vascular permeability, while prostaglandins (PGI₂, PGD₂, PGE₂, and PGF₂α) and histamine contribute to vasodilation (Noli & Miolo, 2001). The signal to start the cellular reaction of the inflammatory phase is when leukocytes flow into the area of the wound. Neutrophils and monocytes are the main cells through the early inflammatory phase at the site of injury. Neutrophils and monocytes start migrating from capillaries into the wounded tissue soon after injury. Neutrophils initially arrive in large numbers, but later in inflammation, neutrophils decline as well as macrophages in number (Li et al., 2007). The most important controlling cell in

the inflammatory reaction is the macrophage as it can phagocytize, digest and kill pathogenic organisms and scavenge tissue debris and destroy any residual neutrophil. Monocytes or macrophages that release enzymatic proteins and biologically active oxygen intermediates permit the initiation of angiogenesis and the creation of granulation tissue (Lewis et al., 1999; White et al., 2004). Another important role of macrophages is to release chemotactic factors which include fibronectin, fibroblast growth factor, vascular endothelial growth factor, platelet derived growth factor, TGF- α and TGF- β . These cytokines are important in encouraging cell migration as well as proliferation which attracts fibroblast to the site of injury and matrix production. Macrophages thus appear as factories for growth factor production and this plays an essential function in the alteration between inflammation and repair (Bosco et al., 2008).

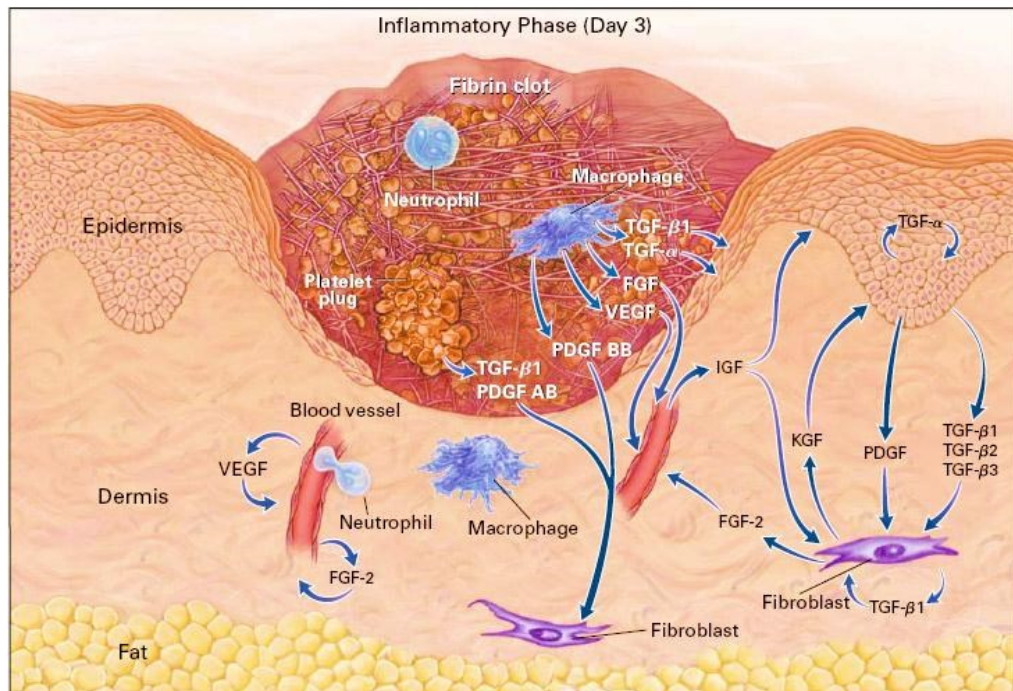


Figure 2.3: Cutaneous wound healing at day 3 after injury. Growth factors thought to be necessary for cell movement and wound healing. In this stage there is expression of TGF-β1, TGF-β2 and TGF-β3. TGF-α, fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), insulin like growth factor (IGF) and keratinocyte growth factor (KGF). Adapted from (Singer & Clark, 1999).

2.2.2.3 Cellular migration and proliferation

After the first week of the acute wound, the cellular medium in the wound varies dramatically. These changes include a heavy infiltration of inflammatory cells to the initial fibrin–fibronectin matrix, while the cells which will predominate as healing progresses are fibroblasts and endothelial cells. Several days after injury, reestablishment of the epithelial surface is initiated for the revascularization of the injured region. Cytokine systems stay designates a portion of this activity as cytokine production shares to fibroplasia, epithelialization and angiogenesis (Hunt et al., 2000). Deactivations of cells via negative feedback mechanisms are also fundamental in the normal healing process (Diegelmann & Evans, 2004). A structural molecule, as fibronectin and collagen of the early extracellular matrix, participates in granulation tissue formation by providing a scaffold for contact guidance and a reservoir for cytokines and growth agents. Collagens

act as a foundation for the intracellular matrix formation within the wound (Baum & Arpey, 2005). Modeling and establishing of new blood vessels is critical in wound healing and this happens simultaneously through all stages of the reparative process, while attracting neutrophils and macrophages through numerous angiogenic factors secreted during the hemostatic phase to promote angiogenesis (Pierce et al., 1991; Takeshita et al., 1994).

2.2.2.4 Protein synthesis and wound contraction

Four to five days after the wound happens (Figure 2.4), the dominant events that start in wound healing are synthesis, deposition of proteins and contraction of wound. The strength of a scar depends on the quality and quantity of deposition during the healing stage (Bullard et al., 1999). Collagen forms more than (50%) of the protein in scar tissue and its production is fundamental for the healing process. Throughout the repair process, fibroblasts are taking charge of synthesis of collagen as well as other proteins (Diegelmann & Evans, 2004).

The numerous cytokines responsible for the stimulation of fibroblasts for collagen synthesis are PDGF, TGF- β and EGF. Various factors related to the patient as well as to the wound influence the rate of collagen synthesis and this includes age, tension, pressure, and stress (Velmar et al., 2009). The synthesis of collagen persists on a top level for 2- 4 weeks and thereafter begins to slow down. Aberration in healing is predominantly the outcome of aberrations in the precipitation of collagen, although there is a variation in the underlying causes. In diabetic patients, there is limited collagen deposition and impaired healing due to impaired inflammatory cell activation, this is associated with a lack in additional features of the healing process (Brem & Tomic-Canic, 2007). As aforementioned, fibrin and fibronectin are composed primarily of the initial wound matrix. The composition of the wound matrix changes due to the acceleration of protein

synthesis in which fibrins such as primary matrix constituents are replaced progressively by collagen and proteoglycans. During this phase, numerous proteins are being actively synthesized such as proteoglycans, which are an essential component of a mature matrix, improving cellular staffing and encouraging wound remodeling by thrombospondin I and SPARC (secreted protein acidic rich in cysteine) (Reed et al., 1993). Four to five days post-wound, wound contraction starts and actively continues for nearly two weeks. Wound contraction persists for longer in wounds which stay open for at least two weeks. Wound contraction is evident in an open wound be closed by wound primes directs closer to each other. Wound contraction in the case of incisional wounds, simply results from limitation of scar and a lesser amount is clear. The percentage of contraction differs according to anatomic sites but it averages from (0.6 to 0.75 mm) per day. The contraction level is also affected by wound shape, such as the case of four-sided wounds that contract more rapidly than round wounds. Round holes subsequently have fewer possibilities of being exposed to risk as they are minor and narrow. The predominance of myofibroblasts at the wound periphery is a cardinal feature of wound contraction. Four to six days after the initial injury, myofibroblasts appear and are usually seen in the wound for 2 to 3 weeks (Porter, 2007). Considering that myofibroblasts are the “motor” that contracts a wound, in the last decade more effort using collagen frames it has been proposed that fibroblasts are a fundamental portion of the wound and may be more essential for wound contraction. TGF- β and probably other cytokines participate in contraction of the wound (Huebener & Schwabe, 2012). All cutaneous wounds heal via three elementary mechanisms: connective tissue matrix deposition, contraction and epithelization. Through the main mechanism of healing during the formation of connective tissue matrix deposition, collagen, proteoglycans and attachment proteins are placed to form a new extracellular matrix; this is for wounds that are simple and can be closed

by tape, sutures or staples and heal faster. In contrast, wounds that continue to remain open essentially heal by contraction. The basic devices accountable for contraction do not fully understand that there seems to be a multifaceted interface between contractile fibroblasts also known as "myofibroblasts" and the matrix constituents (Tomasek et al., 2002). Nerve growth and IL-8 could moderate the contraction response (Iocono et al., 2000).

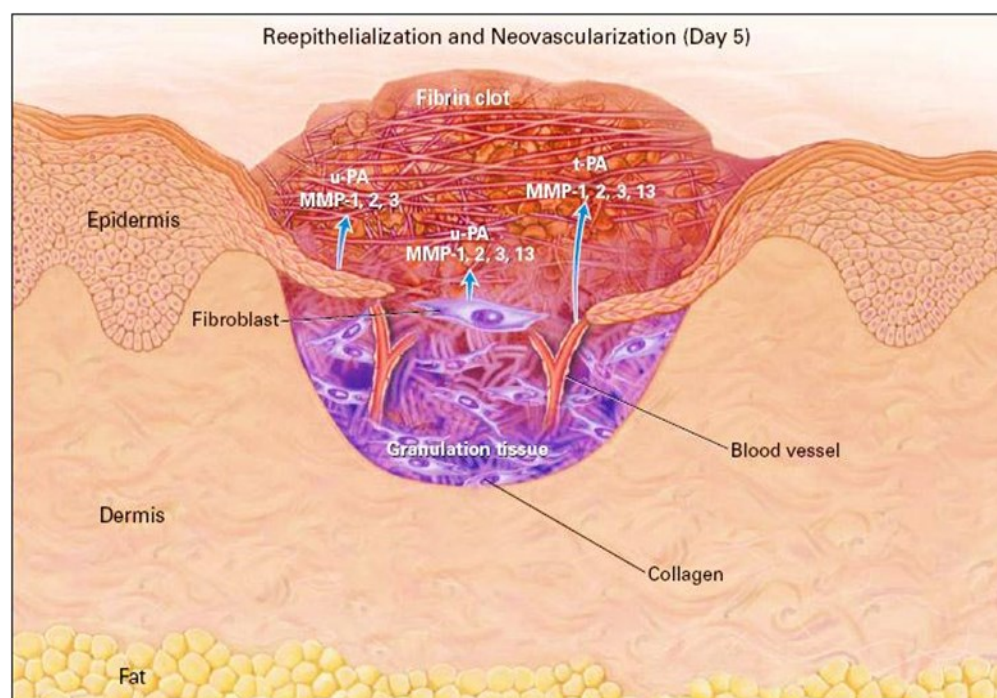


Figure 2.4: Cutaneous wound healing at day five after injury. Blood vessels have appeared to be sprouting into the fibrin clot as epidermal cells resurface the wound. Proteinases, thought to be important for cell movement. In this phase there is expression of urokinase type plasminogen activators (u-PA), MMP-1, 2, 3 and 13 (matrix metalloproteinases 1, 2, 3 and 13) and tissue plasminogen activator (t-PA). Adapted from (Singer & Clark, 1999).

2.2.2.5 Remodeling

Remodeling of the scar starts to predominate by means of the principal wound healing activity about 21 days after the wound (Figure 2.5). The collagen synthesis rate decreases and reaches concurrence with the collagen split-up rate. Interferon- γ , TNF- α , and the collagen matrix itself play a considerable function in the down-regulation of collagen synthesis (Buck et al., 1996; Mori et al., 2002). Matrix metalloproteinases (MMPs) are implicated in a number of roles during wound repair comprising of elimination of non-vital tissue, stimulation of blood vessel growth, keratinocyte migration, regulation of growth factor activity and connective tissue remodeling. MMPs are implicated in the active breakdown of collagen molecules that takes place throughout the remodeling process. Therefore, MMPs are engaged in many aspects of the wound healing process (Gawronska-Kozak, 2011).

Currently 23 MMP genes that break down diverse extracellular matrices have been identified in humans (Visse & Nagase, 2003). MMPs are created via multiple kinds of cell. Different cells commonly manufacture diverse enzymes and the modulation of MMP action inside tissues is via tissue inhibitors of metalloproteinase (TIMPs). There are four isoforms of TIMPs that have been described (Brew et al., 2000). The equilibrium of MMPs and TIMPs inside tissue is pivotal to enzyme action and is controlled through cytokines excluding TGF- β , PDGF and IL-1 (Haroon et al., 2000; Hayden et al., 2011).

TIMP-1 and TIMP-2 are mainly used for wound healing by encouraging cell proliferation, inhibiting apoptosis and preventing matrix MMPs that have been associated in impeding wound healing directly by destruction of extracellular matrix proteins and indirectly through out degradation of growth factors and cytokines (Ching et al., 2011).

Modification of the extracellular matrix composition is one of the features of wound remodeling. Approximately 80% of the dry weight from normal human dermis are the main proteins responsible for strength in collagen fibers, which are responsible for the structure and hardness of dermal tissue. In healthy adults, type I collagen consists of about 80% of all collagen in the dermis and 10% is type III collagen. Early in the repair period, the entire quantity of collagen increases reaching a maximum level between 2-3 weeks after injury. The dermis gradually returns to the normal phenotype, which contains mainly type I collagen after a period of one year or even longer. With wound closure, regular modification occurs to the type III collagen and it undergoes degradation, while type I collagen synthesis increases. Controlled production of new collagen and lysis of old collagen, mainly executed by the actions of MMPs are involved in the process of transformation of the dermis collagen (Li et al., 2007). During the last phase of wound healing, the remodeling phase is in charge of the improvement of the new epithelium and final scar tissue creation. Synthesis of the extracellular matrix in the proliferative and remodeling phases starts with granulation tissue development.

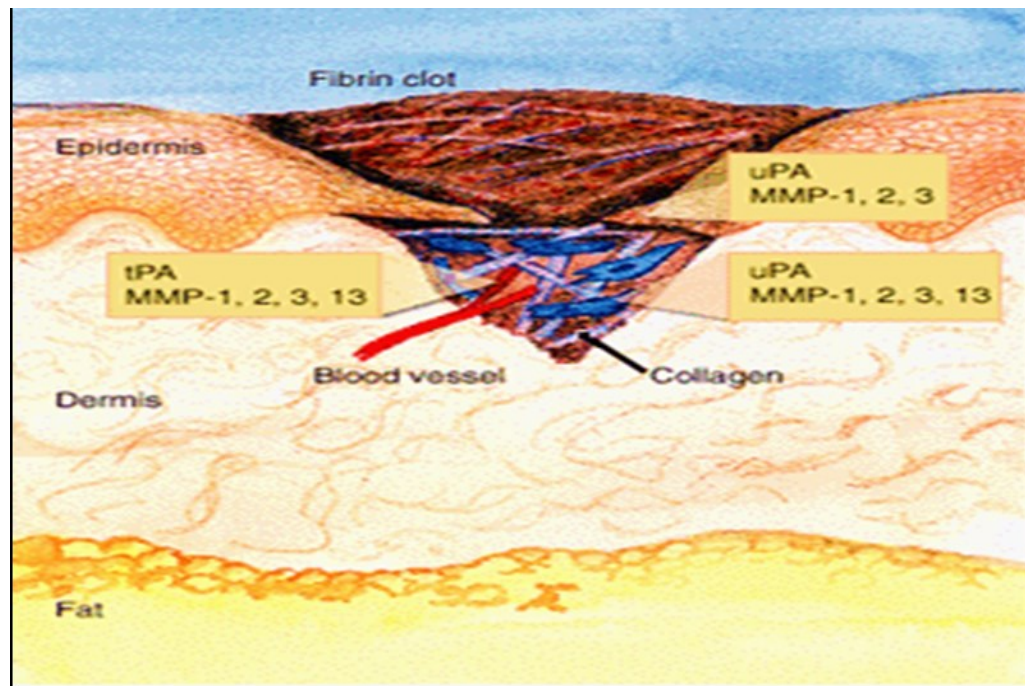


Figure 2.5: Cutaneous wound healing in remodeling phase. The remodeling phase of wound healing is also cytokine-mediated. Degradation of fibrillar collagen and other matrix proteins is driven by serine proteases and MMPs under the control of the cytokine network. Granulation tissue forms below the epithelium and is composed of inflammatory cells, fibroblasts and newly formed and forming vessels. Adapted from: (Wahl, 2002).

2.2.3 Factors effects on wound healing

Local and systemic factors may influence the rate of wound healing. Local factors refer to situations in the direct wound environment whereas systemic factors describe entire body effects on the local wounded area (Guo & Dipietro, 2010).

2.2.3.1 Infection

An infected wound is usually observed as a wound that heals slowly. The presence of bacteria in the wound generates an inflammatory host response. Exotoxins are secreted by living bacteria in the wounded tissue, which can be described as a waste product of bacterial active metabolism. Exotoxins are toxic to the cells of wounded tissue and so inhibit the normal function of local cells and tissues. Highly cleaned and often-systemic antibiotics are needed for infected wounds and foreign bodies found in a wound can be

possibly related to infection (Guo & Dipietro, 2010). Inflammation is a normal phase of the wound healing process and is essential for removing the contaminating microorganisms. The extended elevation of proinflammatory cytokines as TNF- α and interleukin-1 lengthen the inflammatory phase that can be caused by both bacteria and endotoxin. The wound may come to a chronic stage and fail to heal if this remains. The extended inflammation can be an indication for increasing the level of MMPs a family of proteases, which could reduce the extracellular matrix (Menke et al., 2007).

2.2.3.2 Local blood supply

Cell metabolism, particularly energy production, is crucial for almost all wound healing processes and needs oxygen. It preserves infection of wounds from angiogenesis, increasing keratinocyte differentiation, migration and re-epithelialization, improving fibroblast proliferation and collagen production, and encourages wound contraction (Bishop, 2008; Rodriguez et al., 2008). Furthermore, the level of superoxide is the key to killing microorganisms produced through polymorphonuclear leukocytes, and it is dependent on oxygen levels. Impermanent hypoxia activates wound healing, nevertheless extended or long-lasting hypoxia interrupts wound healing. Hypoxia aids as a signal in acute wounds, which encourages many aspects of the wound healing progression and could prompt cytokine and growth agents produced from keratinocytes, fibroblast and macrophages. This includes, TGF- β , platelet derived growth factor, TNF- α , vascular endothelial growth factor and endothelin-1 which are all important promoters of cell proliferation, migration, chemotaxis and angiogenesis in the wound healing process (Tandara & Mustoe, 2004).

2.2.3.3 Malnutrition delays healing

Proteins are the chief building blocks for cell renewal, growth of tissue and restoration post damage. They significantly influence numerous wound healing phases through their part in DNA and RNA production, collagen and elastic tissue formation, nutrition of the immune system, epidermal growth and keratinization. With continued protein malnutrition, skin will be thinner, more wrinkled and immunity decreases. Diabetic patients are exposed to a higher threat of damage with protein malnutrition (Dryden et al., 2013). Adequate protein intake is fundamental to wound healing and a delay in wound healing in the case of protein depletion appears to be due to extending the inflammatory phase through inhibited proteoglycan and collagen production, inhibition of fibroplasia and inhibition of neoangiogenesis via inhibited wound remodeling (Ruberg, 1984).

Dietary proteins that supply all nine of the necessary amino acids are assumed complete proteins. Poultry, meat, fish, milk products, eggs and soyabeans are considered good sources of complete protein. The body needs a sufficient amount to provide necessary amino acids, adequate nitrogen and energy for the production of the eleven other amino acids. There is an indication that many amino acids are particularly essential to the wound healing process. Cysteine and methionine are implicated in the production of collagen and connective tissue. The chief effect on the proliferation of collagen and enhanced immune reaction is from arginine (Wild et al., 2010). Glutamine is also important in the wound for the proliferation of inflammatory cells and as a source of energy (Dryden et al., 2013).

Carbohydrates are a fundamental source of energy rich foods. Carbohydrates are essential to fuel the metabolic process in the cells and tissues involved in wound healing. Because of this we need an increased intake of energy giving foods in patients with extensive wounds. As soon as adequate carbohydrates are ready-made, the body does want

to break amino acids for energy production (Medlin, 2012). Glucose deficiencies affect fibroblast proliferation, and significantly, lower glucose levels have been noted in the wound fluid of chronic wound patients (Han et al., 2004).

Fats are constructed of sub-units called fatty acids; these fatty acids are composed of component cells and tissues (cell membranes), so a supply of fatty acids is required for tissue regeneration. Fat provides the necessary fatty acid building blocks and acts as a source of energy for cell metabolism (Shingel et al., 2008).

Vital micronutrients in the diet are vitamins (vitamins A, D, E and K), which are fat-soluble and may be stored in the body while vitamin B1-B11 and C are water-soluble and cannot be stored. A fundamental cofactor for the synthesis of proteoglycans, collagen and other organic components of the intracellular matrix of tissues, skin, capillary walls, bones and other connective tissues, is vitamin C (ascorbic acid). Ascorbic acid deficit causes irregular collagen fibers and changes in the intracellular matrix that are evident in cutaneous lesions, decreased tensile strength of fibrous tissue, poor adhesion of endothelium cells and poor immunity (Biesalski, 2010; da Rocha et al., 2002).

The vitamin essential for epithelial and bone tissue development, immune system function and cellular differentiation is Vitamin A. Vitamin A converses corticosteroid and induces the inhibition of cutaneous and fascial wound healing as well as enhancing wound repair in the normal physiological process. Wound through facilitated the early inflammatory phase, involving improved localization and stimulation of the immune response, increased number of macrophages and monocytes in the wounded tissue, support of epithelial cell differentiation and modulation of collagenase activity, were enhanced by vitamin A (Mackay & Miller, 2003).

Inorganic nutrients required in small quantities for health are minerals. Zinc is an essential mineral needed for wound healing, and this is perhaps important for the re-

epithelialization process. Around three hundred enzymes require zinc for their action and zinc is fundamental for cell division, DNA synthesis and protein synthesis, 45 as well as all necessary procedures for tissue renewal and repair. Deficiencies in zinc have been related to delayed wound healing and the decreased break strength in animal wounds that comes from decreased collagen and protein production throughout the healing process in zinc-deficient animals (Prasad, 1995).

A deficiency of zinc is accompanied by an immunodeficiency causing an increased number of infections. Zinc has an effect on keratinocytes, osteocytes and leukocytes, and different concentrations of zinc are required for a specific cellular response (Rink & Gabriel, 2001). A cofactor of enzymes (prolyl and lysyl hydrolysis), which are important for the synthesis of collagen, is iron. Therefore, a greater iron deficiency strongly impedes the wound healing process. In addition, iron as a part of hemoglobin plays an important part in the oxygen transport to regenerate wounded tissue (Brown & Phillips, 2010).

Water is also crucial for optimal healing as cell proliferation and migration sideways chemotactic gradients formed by cytokines, metal ions and growth factors are encouraged by hydration. Dehydration causes epidermal hardening and dermal necrosis that delays healing and adds to patient discomfort (Dryden et al., 2013).

2.2.3.4 Age effects

Young adults and children generally heal well. Wounds need all the necessary nutrients for wound healing as well as the conventional requirements for growth and development. Wound healing may be slow due to a small amount of fibroblasts in the tissues and this consequentially reduces amounts of collagen creation in the elderly. The contraction of the wound and re-epithelialization is slower in older people. The underlying disease processes in the elderly may adversely affect wound healing as in cases of pe-

ipheral ischemia, heart disease and diabetes mellitus. More sustainably, to have reducing mobility with increasing risk of pressure sore formation is elderly (Gosain & Dipietro, 2004; Oriana et al., 2012).

2.2.3.5 The reactive Oxygen Species

The process of wound healing is controlled by a selection of disparate cytokines, growth factors and hormones. Continuing studies have shown that nitric oxide and reactive oxygen species (ROS) are pivotal regulators of wound healing (Schwentker et al., 2002). ROS is needed for protection from microbial invasion, and intracellular signal transduction also requires a low level of ROS. For example, hydrogen peroxide at low levels is essential for efficient angiogenesis of the wound. However, excessive ROS is poisonous because of the high reactivity. Both hyperoxia and hypoxia increase ROS formation but a raised level of ROS exceeds the beneficial influence and results in additional tissue damage (Guo & Dipietro, 2010). During the course of normal metabolic processes, for example, respiration, ROS is produced by all cells. In wounded and inflamed tissues, inflammatory cells produce high levels of NADPH oxidase that in turn produces a particular level of ROS (Schreml et al., 2010).

Cells form highly reactive superoxide radical anions, which are rapidly dismutated to hydrogen peroxide (H_2O_2) and water. This is enhanced by many superoxide dismutases. Even though, H_2O_2 is not a radical, it can cause serious cell injuries because of the generation of hydroxyl radicals in the presence of iron or copper. Hydroxyl radicals are a highly aggressive factor resulting in the oxidation of cellular macromolecules, and for this reason, H_2O_2 must be quickly detoxified and this is achieved by catalase and various peroxidases. If the production of ROS occurs in an excessive amount or in the case of unsuccessful detoxification, oxidative stress occurs, these results in serious cell damage, premature aging or even neoplastic transformation (Steiling et al., 1999). ROS is

formed in high amounts at the site of the wound as a protection mechanism against invading bacteria (Srinivas et al., 2008). However, the existence of increased numbers of neutrophils and ROS overpowers the anti-protease substances that normally defend the tissue cells and the extracellular matrix. At high concentrations, ROS can prompt severe tissue injury and even cause neoplastic transformation and reduce the healing process via damage to the cellular membranes, proteins, DNA, and lipids (Ipek et al., 2012). Excess ROS results in the killing of fibroblasts, and skin lipids will be less flexible. Because of this, the general role of antioxidants seems to be significant in the effective treatment and management of wounds. Antioxidants diminish the adverse effects of wounds through eliminating products of inflammation (Houghton et al., 2005). The most probable mechanism for antioxidant defense is the direct interface of the extracts or compounds and hydrogen peroxide, instead of changing the cell membranes and controlling damage. Compounds have a high radical scavenging capacity shown to enhance wound healing (Ipek et al., 2012). Numerous low molecular weight antioxidants suggest regulating the redox environment in skin wound healing. Those involved in the endogenous molecules are glutathione, uric acid and lipoic acid, in addition to compounds found in food such as vitamins C and E, carotenoids and phenolic compounds. The useful significance of these antioxidants in wound healing is confirmed by their depletion in healing skin wounds (Mackay & Miller, 2003).

2.2.4 Role of apoptosis in wound healing

In the process of wound healing, some probable signals for the expulsion of cells involved in tissue repair have been proposed but the mechanism by which the cells die is still required. Emigration, necrosis and apoptosis are three probable mechanisms. Pathological tissue repair, necrosis happens, but for most wounds, healing proceeds without an exaggerated inflammatory response to tissue injury. Tissue inflammation is obviously induced by necrosis and so the mechanism that reduces wounds cellularity is possibly not controlled by necrosis (Greenhalgh, 1998). There is little evidence to support cellular migration away from the wound, which would be wasteful of energy and inefficient. Apoptosis is a reasonable mechanism for the diminishing number of cells throughout the healing phases. The elimination of undesirable cells and tissues are commonly via apoptosis in a phagocytotic process that does not elicit an inflammatory response. Subsequently, removal of dead tissues and invading pathogens and inflammatory cells would be expected to diminish and disappear from the wounded area without inciting more inflammation via apoptosis. Similarly, when appropriate deposition of collagen, latterly of the proliferative phase, takes place, fibroblasts would begin to be apoptotic. Ultimately, after maturation of the wound is completed a slow disappearance of endothelial cells and remaining fibroblasts will take place. There is much evidence of an association between apoptosis and the resolution of tissue repair in the wound healing process. For example, in one study, during the early phase of tissue repair the inflammatory cells experienced apoptosis as early as 12 hours after wounding (Singer & Clark, 1999). There was a clear apoptosis in the inflammatory cells localized under the margin of the migrating epithelium and immediately next to the wound margin. The granulation tissue wrapped with an epithelium had no apoptosis and minimal inflammatory cell infiltration, and auxiliary fibroblasts were observed in the open dermal wound, covered by

a polyurethane dressing, of non-diabetic and diabetic mice (Darby et al., 1997). In the center of the wound, the open granulation tissue was packed with neutrophil, macrophage and lymphocyte inflammatory cells. Apoptosis was observed in the altered region which was under a location where the lead margin of epithelium had migrated across the granulated tissue. This observation confirms the hypothesis that something in the epithelium is responsible for the inhibition of inflammation. These observations are supported by clinical findings. Closure of a wound, firstly, or covering with a graft is associated with the start of the elimination of inflammatory cells from the wounded tissue (Ashcroft et al., 2003; Rodero & Khosrotehrani, 2010). The function of apoptosis in the elimination of fibroblasts in the later stages of tissue repair is through reducing cellular infiltration throughout the switch between granulated tissue and scar. If the signal responsible for down-regulation of fibroblast and myofibroblast action is late and after a specific time then the apoptotic process is permanently depressed. Excessive fibroblast activity is caused by an imponderable between collagen synthesis and degradation leading to formation of an extreme scar. Until now, the exact mechanisms contributing to excessive scar formation are unknown (Desmouliere et al., 1995).

Happily, apoptosis is the foundation in the control of the perfect neutrophil response to injury so that major wounds do not result in unhelpful tissue responses. After the elimination of invading pathogens in the wound tissue, most neutrophils undergo apoptosis and are speedily and efficiently phagocytosed through macrophages without eliciting further inflammation (Rodero & Khosrotehrani, 2010). Multiple possible mechanisms of neutrophil apoptosis are postulated and neutrophil apoptosis may be induced via the TNF- α signaling pathway (Ohta et al., 1994), interaction of neutrophils with the $\beta 2$ integrins (CD11b/CD18) potentiating the extent of apoptosis and migration of neutrophils by a monolayer of endothelial cells and induced apoptosis (Larsson et al.,

2000). TGF- β 1 has a high likelihood of being implicated in the down-regulation of the inflammatory response. Evidence supporting its function in removing inflammatory cells comes from TGF- β 1 null knockout animals (Desmoulière et al., 1997). The absence of TGF- β 1 expression in mice is characterized by a normal healthy status until 3 weeks of age when the pups suffer from a deadly inflammatory response categorized by invasion of neutrophils, macrophages and lymphocytes into all tissues. Reaction is related to elevated expression of TNF- α and IL-1 β . Repair of tissue in these pups was normal throughout the first three weeks due to the knockout animals receiving TGF- β 1 through their mother's milk and was stopped in the period of weaning when the animals became ill from inflammatory cell invasion (Sporn & Roberts, 1992).

The antiproliferative protein p53 is also included in the apoptosis of inflammatory cells during the healing process (Fuchs & Steller, 2011). The control of keratinocyte apoptosis has attracted more interest in dermatological diseases. Exposure of keratinocytes to ultraviolet light (sunburn) leads to rapid induction of cell apoptosis (Raskin, 1997). Eighteen member proteins of the BCL2 family have been discovered, which are effectors of the apoptosis pathway. The BCL2 family proteins either promote or prohibit apoptosis. The pro-apoptotic proteins include BAX, Bad and Bak, while BCL2 and BCL-xL are anti-apoptotic proteins. The lack of basement membrane connection is associated with a decrease in BCL-xL expression and induction of apoptosis. Similarly, EGF receptor blockage has been associated with apoptosis and reduced BCL-xL expression (Rai et al., 2005). Penal et al. looked at the function of BCL-xL, which keeps down apoptosis and BCL-xS, which stimulates apoptosis in transgenic mice. They found that over-expression of BCL-xL or BCL-xS in mice shows that the keratinocytes have a normal appearing epidermis. Post ultraviolet light irradiation, mice with BCL-xL over-expression had mounting resistance to damage while those with BCL-xS over expres-

sion had mounting sensitivity. Disparate pathways for keratinocyte apoptosis in injury and in response to normal differentiation have been proposed (Pena et al., 1997).

A decline in p53 levels in keratinocyte by siRNA treatment improved, instead of reduced, apoptosis responses to interferon- α and interferon- γ . Results show over exposure of the human keratinocyte cell line to interferon-stimulated apoptotic. The mechanism for this boosted apoptosis complicates the stimulation of TRAIL and its interaction with death receptors, as obstructing the death receptor pathway using dominant negative FADD or by the addition of neutralizing antibody versus TRAIL, decreases the apoptosis response to IFN- α and IFN- γ (Chaturvedi et al., 2006).

The range of hypertrophic or keloid scarring is decreased by interferon and IFN- γ initiates to diminish the range of keloid formation (Durani & Bayat, 2008; Granstein et al., 1990). Systemic treatment of burn patients using IFN- α 2b was initiated to decrease hypertrophic scar creation (Harrop et al., 1995). Elevated TGF- β serum levels were found in patients that tended to have hypertrophic scars (Schmid et al., 1998). This suggests that fibroblast activity is regulated by an interface between interferon and TGF- β in a maturing wound. Collagen degradation is also regulated by apoptosis signals via induced collagenase action. Bian and Sun commented that p53 binds to the promoter of human type IV collagenase (matrix metalloproteinase 2) (Bian & Sun, 1997). They proposed that apoptosis signals are implicated in the down-regulation of collagen deposition by both diminishing fibroblast numbers (thus decreasing collagen synthesis) and by stimulating collagenase activity. A previous study on the effects of recombinant human TNF- α on collagen production and gene expression in cultured fibroblasts proved that TNF- α reduction transcription of the collagen gene, expression of collagen mRNA levels and collagen production in cultured fibroblasts (Solis-Herruzo et al., 1988).

Cellularity and the extracellular matrix in granulation tissue found in the late phase of wound healing decreases to produce a mature scar. It has been proposed that apoptosis is a mechanism facilitating the conversion from granulation tissue to scar. Nakazono-Kusaba, et al. investigated the influence of benzoyl staurosporine, a protein kinase C inhibitor on human keloid derived fibroblasts to study if this agent is capable in the treatment of keloid formation. The results show that benzoyl staurosporine could be a candidate for antitumor factors by prompting keloid fibroblast apoptosis throughout caspase dependent pathway (Nakazono-Kusaba et al., 2004).

In conclusion, apoptosis plays a critical role in the synchronization of the rapidly altering residents of cells, which are implicated in the healing of all tissues. In massive common of wounds, control of proliferation causes an accelerated closure of the wound. Sometimes balancing increasing and decreasing cellular numbers forfeits the balance and leads to pathologic tissue repair.

2.3 Immunomodulatory

The immune system is a remarkably developed defense system inside vertebrates which guards against attacking factors. It is capable of producing diverse molecules and cells able to distinguish and reduce unlimited changes from external and unwanted agents. Modulation of the immune system refers to some alteration in the immune response which includes stimulation, amplification, expression or inactivation of some stage of the immune response. Therefore, an immunomodulator is a material used to have an effect on the immune system. There are commonly two kinds of immunomodulator depending on their influence: immunostimulators and immunosuppresses (Saroj et al., 2012). Materials, which show a modifying immune system response to a threat, are immunomodulators. They modulate and arm the immune system by keeping them in a highly prepared state against any threat (Mishra et al., 2008). In different parts of the world, plant extracts have been widely investigated for their possible use for immunomodulation. Many studies have demonstrated isolation of the potential bioactive molecule (Alamgir & Uddin, 2010). For example, *Acorus calamus* a rhizome extract exhibited growth of many cell lines for humans and mice. It could also inhibit the formation of interleukin-2 (IL-2), nitric oxide (NO) and tumor necrosis factor- α (TNF- α). In addition, it causes down-regulation expression of the CD25 marker (Mehrotra et al., 2003) and many other plant derived compounds like sterols, sterolins, polysaccharides, alkaloids, flavonoids, lectin and glycoprotein are used for immunomodulation (Kolm et al., 2007; Lien et al., 2003).

2.3.1 Immunomodulatory dietary

There are many ways immunomodulation resists a huge range of human and animal diseases such as cancers, viral diseases, inflammatory conditions and autoimmune diseases. Living and non-living factors surrounding us interact with the immune system. A diet should be balanced with its components coming from vitamins, trace minerals and energy. All these have a critical effect on the immune system, starting from their active role in developing immunity, therefore any deficiency predominantly ends in disease. Both living and non-living factors are able to work as immunomodulators. The living factors include many infectious agents such as different plants, parasites, protozoa, fungi, bacteria and viruses, whereas non-living factors include microparticles and food additives such as titanium dioxide and aluminium silicate. Other non-living factors which co-contribute to the immune system response are environmental factors like air, temperature, radiation, water, pressure, food and toxins (Mahima et al., 2013).

Microparticles are small, non-biological particles that are used in numerous areas of daily life as food additives. The most common food-derived ingested compounds are titanium dioxide and aluminium silicate. Titanium dioxide is a white pigment used in sugar toppings or toothpaste. Becker et al. (2013) proved that macrophage-like cells easily take up titanium dioxide after a 6 h incubation period with titanium dioxide resulting in the assembly of caspase-1 and increased secretion of IL-1 β . In intestinal epithelial cells, titanium dioxide microparticles may be swallowed by macrophages and intestinal epithelial cells and induce IL-1 β and IL-18 secretion. This may trigger inflammation in susceptible individuals (Becker et al., 2013). Wischke et al, showed the effect of special carriers from poly rac-lactide-co-glucolide to particulate which is to prompt a dose dependent maturing of human monocyte derived dendritic cells to a proinflammatory phenotype with a high level of released cytokines (Wischke et al., 2012). Pre- pro- and syn-

biotics enhance innate immunity. Several plant fibers (prebiotics) have recognized significant effects, which are to increase the function of the innate immune system and the physical barrier in order to increase resistance to disease (Bengmark, 2012).

The food that we eat is fundamentally composed of proteins, carbohydrates, fats, minerals and vitamins. A suitable integration of these materials is essential to preserve the health of humans. Fats have a potential role in manipulating the immune response. Fatty acids in the metabolism process are changed to powerful biological mediators, which play a crucial role as immunomodulators. For example, linoleic acid converts in the individual body to arachidonic acid in the plasma membrane of the immune cell and produces leukotrienes and prostaglandins, which have an important role during inflammation (Calder & Grimble, 2002).

Proteins are an essential source of amino acids, important in building cells, and have a critical role in the immune system. Antibodies are proteins in origin, arginine is important in the activation of myeloid cells and includes many different cellular types, from polymorphonuclear to monocytes/macrophages (Peranzoni et al., 2008). In one study, arginine and glutamine were given as supplementation to a macrophage culture media and, the results showed that this enhanced macrophage phagocytosis and killing ability and proliferation of T- and B-lymphocytes significantly. These results suggest that *in vitro*, arginine and glutamine are essential substrates and immunomodulators of both innate and adaptive immunity responses in fish leukocytes (Pohlenz et al., 2012). Vitamins are crucial for the normal body's defense system through their various roles in the immune system. Vitamin A, B, C, D and E have been proven in previous studies to be important in improving both the innate and adaptive immune response (Mahima et al., 2013). Minerals like vitamins have a main function in the modulation of the immune response. A deficiency of copper cause decreases in interleukin2 and T cell proliferation

(Percival, 1998). Selenium deficiency in the diet results in the reduction of macrophage stimulation (Ren et al., 2012). In conclusion, immunity is the defense system of the body. Its optimum function depends on essential factors which can be supplemented by diet and the balance between these factors are important to maintain an optimal function of the immune system.

2.3.2 Recent improvements on the ethnomedicinal plants as immunomodulatory agents

Modulation of the immune response to reduce diseases has long then been of interest and there have been many studies on ethnomedicinal plants as immunomodulatory agents. Immunopharmacology is a relatively innovative and developed branch of pharmacology which aims to search for immunomodulators. The possible uses of immunomodulators in clinical medicine include reconstruction of immune deficiency, for example, in the treatment of AIDS and suppression of normal or exaggerated immune roles as in the treatment of autoimmune diseases. An important source of immunomodulators is medicinal plants and their active components. Therefore, the improvement of drugs for immunomodulation and anti-tumor potential from natural compounds is an interesting project (Alamgir & Uddin, 2010).

2.3.2.1 Immunomodulatory activity of crude plant extracts

Previous studies have demonstrated that many of the plant crude extracts have immunomodulatory activity. Ethanol extract of the *Acorus calamus* rhizome has immunosuppressive potential *in vitro*. It inhibits proliferation and encourages human peripheral blood mononuclear cells (PBMCs) to produce interleukin-2, TNF- α , IFN- γ , nitric oxide and expression of cell surface markers CD16 (Mehrotra et al., 2003).

Crude extract of *Tinospora cordifolia* produces a polyclonal B cell mitogen that improves immune response in mice. Arabinogalactan polysaccharide isolated from the stem of *T. cordifolia* was tested to modulate induction of immunosuppression. Mice pre-treated with arabinogalactan polysaccharide showed protection against lipopolysaccharide (LPS) prompted mortality and increased serum interleukin-1 β , interleukin -6, IFN- γ levels and decreased serum interleukin-10 compared to the controls (Desai et al., 2007). Other studies investigated the effect of polysaccharide isolated from *T. cordifolia* on phenotypic and functional maturation of murine bone marrow derived dendritic cells. Results showed enhancement of surface expression of CD80, CD40, CD86 and MHCII on derived dendritic cells and stimulated T cells for the secretion of TNF- α and interleukin-12 (Pandey et al., 2012). Ethanol extract of *Boerhaavia diffusa*, a plant used in Indian traditional medicine, significantly suppressed human NK cell cytotoxicity *in vitro* and inhibited production of nitric oxide in mouse macrophage cells, interleukin-2 and TNF- α in human PBMCs. The results demonstrated the immunosuppressive potential of ethanol extract of *B. diffusa* (Mehrotra et al., 2002).

2.3.2.2 Immunomodulatory activity of plant derived compounds

The β -sitosterol, phytosterols, and its glucoside, were investigated *in vitro*. There was an enhanced proliferative response of T-cells activated by sub-optimal concentrations of phytohaemagglutinin which significantly improved the expression of CD25 and increased the secretion of interleukin-2 and TNF- γ (Bouic et al., 1996). Although most studies have focused on the effect of phytosterols on cholesterol-lowering activity one study on phytosterols showed a modulatory effect on the T-helper immune response *in vivo* using a mouse model which caused increased interleukine-2 and IFN- γ secretion (Calpe-Berdiel et al., 2007).

Polysaccharides from plants have prompted researchers to study their physical properties and industrial uses depending on their properties. Over the past 20 years there has been an increase of interest in the biological activity of biomolecules that has led to new sources of bioactive plant polysaccharides (Smestad, 2001). Botanical polysaccharides exhibit a number of advantageous therapeutic properties, and it is assumed that the mechanisms implicated in these effects are through the modulation of innate immunity and more specifically macrophage function. Moreover, botanical and microbial polysaccharides react with common surface receptors and stimulate similar immunomodulatory responses in macrophages; this suggests that evolutionarily covered polysaccharide structural properties are collaborating with these organisms. Thus, the development of botanical polysaccharides provides a unique chance for the discovery of new therapeutic agents and adjuvants that exhibit advantageous immunomodulatory properties (Schepetkin & Quinn, 2006).

Lee et al investigated the immunostimulatory activity of polysaccharides from Cheonggukjang in RAW264.7 macrophage cells and in an animal model. The results showed that Cheonggukjang polysaccharides stimulate mRNA expressions of inducible

nitric oxide synthase and TNF- α . These results suggested that the Cheonggukjang polysaccharide has a potential function in promoting immunity through its regulatory effects on immunological parameters, nitric oxide, TNF- α productions and alterations in indicators related to stress (Lee et al., 2013).

The polysaccharides from peduncles of *Hovenia dulcis* and their bioactivity potential was studied *in vitro* for immunostimulatory activity. Analysis revealed that they could significantly induce the proliferation of splenocytes and encourage phagocytosis and the nitric oxide production activity of peritoneal macrophages. These results suggest that polysaccharides have effective immunostimulatory activity and could be explored as potential natural immunomodulatory agents (Wang et al., 2012a). Other studies have proved that the *in vivo* immunostimulatory activity of polysaccharide from *Cipangopaludina chinensis* could significantly increase the spleen and thymus signals and enhance the macrophage function. These findings suggest that polysaccharides have a potential immunostimulatory activity and could prove to be potential natural immunomodulatory agents (Xiong et al., 2013). A polysaccharide-enriched fraction isolated from *Curcuma longa* exhibited stimulatory effects on PBMC proliferation and cytokine production (Yue et al., 2010).

Alkaloids and flavonoids are the main components in plants. Various studies around the world have mentioned the bioactivity of these components and their immunomodulatory activity. Berberine, a chief alkaloid constituent of *Coptidis rhizoma* has an anti-inflammatory, antibacterial action, and an antitumor and anti-motility effect. A previous study investigated the effect Berberine had on the stimulation of macrophage RAW264.7 cells in LPS stimulation. This alkaloid caused suppression of nitric oxide gene expression that resulted in the reduction of nitric oxide protein production. Besides the production of PGE2 in LPS stimulated RAW264.7 cells was significantly reduced.

while there was increased creation of IL-12 in LPS stimulated RAW264.7 cells macrophages (Lee et al., 2003).

Tripterygium wilfordii hook f. is one of the traditional Chinese medicines that is usually used to treat rheumatoid arthritis. The total alkaloids are the constituent part of *T. wilfordii* hook f. Zhang et al studied the effects of the total alkaloids of *T. wilfordii* hook f. used to inhibit the symptom of type II collagen induced arthritis in wistar rats. It was found that the alkaloids could significantly inhibit the symptoms of type II collagen induced arthritis and reduce the production of IL-6, IL-8 and TNF- α in serum and the expression of IL-6, IL-8, nuclear factor kappa β (NF- κ B) and TNF- α in synovial tissue, thus targeting the inflammation. These results show that alkaloids could be used as a novel botanical drug for the treatment of rheumatoid arthritis (Zhang et al., 2013b).

Flavonoids are polyphenols regularly taken in the diet and have been suggested to perform a number of helpful activities for human health including anti-inflammatory activities. López-Posadas et al. selected nine flavonoids to distinguish their effects on leukocyte viability, proliferation, expression of cyclooxygenase 2, inducible nitric oxide synthase and proinflammatory cytokines (TNF- α , INF- γ and IL-2). Inducible nitric oxide synthase was inhibited largely by several flavonoids, especially luteolin, quercetin and apigenin, while cell viability was promoted by kaempferol, chrysin, daidzein and hesperetin. All flavonoids had powerful antiproliferative effects. These results show that flavonoids have a powerful effect on lymphocytes (López-Posadas et al., 2008). Propolis flavone could significantly stimulate lymphocyte proliferation, improve antibody titer and the concentrations of IgM and IgG and significantly increase the immune organ indexes and increase concentrations of IL-2 and IL-6 in serum (Fan et al., 2013).

In conclusion, immunomodulation using medicinal plants could supply alternate predictable chemotherapy for different diseases, particularly once there is a weakened im-

mune response and when discriminatory immunosuppression happens, as in the case of autoimmune syndromes. There is intense activity to detect additional particular immunomodulators that imitate or antagonize the biological properties of interleukins and cytokines. Improvement of evaluation of these mediators will create sensitive and specific screens. We should reconsider natural medications that could be important sources of innovative ligands capable of directing specific cellular receptors.

2.4 Medicinal plants

Throughout history, plants have supplied humans with a source of food, perfume, dyes, gum, fiber, resin and many other useful products. Nowadays, ethno pharmacologists are paying more attention to investigating the bioactive properties and phytochemical analysis of medicinal plants to treat a variety of diseases. Numerous medicinal plants have significant therapeutic functions and could be used as natural medicine sources for the treatment of a variety of diseases (Gupta & Sharma, 2006). Recently, many researchers have endorsed a traditional belief in utilizing medicinal plants to treat a variety of diseases including gastric ulcer. Many studies have reported that plants offer various significant bioactivities (Alama et al., 2009; Yadav et al., 2011).

Phaleria macrocarpa and *Tinospora crispa* are traditional drugs that are used in the treatment of inflammation, cancer and diabetes (Hendra et al., 2011a; Ihsan et al., 2011). In this work, we have used *P. macrocarpa* fruit and *T. crispa* stem extracts to investigate the toxicity of the plants and to investigate their ability in gastro protective, wound healing and their immunomodulatory potential.

2.4.1 *Phaleria macrocarpa*

2.4.1.1 Taxonomy and systematics in plants

Kingdom: Plantae

Division: Spermatophyta

Sub division: Angiospermae

Class: Dicotyledoneae

Order: Thymelaeales

Family: Thymelaceae

Genus: *Phaleria*

Species: *Phaleria macrocarpa* (Scheff) Boerl

There are many different names for the plant; ‘crown god’ and *P. papuana* in an area known as the Malay simalakama, while in Java, it is known as *mahkota dewa*, *mahkuto mewo*, *mahkuto ratu* and *mahkuto rojo* (Susilawati et al., 2012). Its original habitat is New Guinea and Papua Island, Indonesia and other tropical areas. *P. macrocarpa* grows throughout the year in tropical areas reaching a height of 1-6 meters. It is a complete tree (stem, leaves, root, flower and fruit) and the fruit shape is eclipse with a diameter of around 3cm. The color of the fruit is green before ripening and red when fully ripe (Figure 2.6) (Hendra et al., 2011b).



Figure 2.6: *Phaleria macrocarpa* ripe fruit.
Cited by: http://id.wikipedia.org/wiki/Mahkota_dewa

2.4.2 *Tinospora crispa*

2.4.2.1 Taxonomy and systematics in plants

Kindom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Ranunculales

Family: Menispermaceae

Genus: *Tinospora*

Species: *Tinospora crispa*

Tinospora crispa is known by various vernacular names such as *akar seruntum*, *Menispermum crispum* (Linn.) and *Menispermum rimosum* (Blanco). Commonly known by the Malays as *bratawali*, *andawali* and *putrawali* or *akar patawali*. *T. crispa* is an indigenous plant and can be found distributed from the southwestern part of China to the southeast of Asia including Malaysia. It is a climber plant and can be found in tropical and subtropical India, parts of the Far East and in primary rainforests or mixed deciduous forests. The plant is woody with a shiny green leaf (Figure 2.7) and is widely distributed throughout Indonesia, Malaysia, Thailand and Vietnam (Dweck & Cavin, 2006).



Figure 2.7: *Tinospora crispa* stem and leaves.

Cited by: <http://home.hiroshima-u.ac.jp/shoyaku/Wam2003.html>

2.4.3 Ethnopharmacology

Ethnopharmacological evidence shows numerous uses of *P. macrocarpa* and *T. crispa* by humans. The main purpose of these plants is as a source of food additives and as a natural source of medicinal agents. (Mohammed et al., 2012). A wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, fatty acid, carbohydrate, benzophenone glycosides and steroids are rich in these plants, which have also been screened *in vitro*, and the indicated antioxidant, anti-inflammatory and anti-microbial properties are used to develop drugs or dietary supplements (Simanjuntak, 2008; Yosie et al., 2011).

2.4.3.1 Ethnopharmacology of *Phaleria macrocarpa*

Several studies have investigated the ethnopharmacology usage of both *P. macrocarpa* as a crude extract or through isolated fraction and active compounds from various parts of the plant. Chong et al., 2011 investigated, both *in vivo* and *in vitro*, the effects of *P. macrocarpa* fruit on the control of body weight and the systematic basis of its antihypercholesterolemic effect. *P. macrocarpa* extract significantly reduced body weight gain, triglycerides, total cholesterol and up-regulated the hepatic LDL receptor as well as PCSK9 proteins in hypercholesterolemia rats. These results were confirmed by testing the effect of *P. macrocarpa* extract on HepG2 cells. The extract also significantly up-regulated both PCSK9 at the mRNA and protein level and the LDL receptor. This study proved the potential usage of *P. macrocarpa* fruit for regulating body weight of obese people and treating hypercholesterolemia. *P. macrocarpa* fruits have various amounts of total phenolic and flavonoid compounds. In the study, different parts of the fruit of *P. macrocarpa* (pericarp, mesocarp and seed) showed high antioxidant, anti-inflammatory and cytotoxic activities (Hendra et al., 2011a). Administration of *P. macrocarpa* fruit extract to Sprague Dawley rats for 7 days significantly reduced blood

pressure (Yanti & Ringoringo, 2010). There has been much research on *P. macrocarpa* for its anticancer activity. The *P. macrocarpa* seed has been used experimentally for treating cancer. The ethanol extract of the *P. macrocarpa* seed has been shown to inhibit the proliferative breast cancer cell line T47D (Bakhriansyah, 2012). *P. macrocarpa* bark extract has an anti-proliferative effect against human cancer cell lines Hela, Thp-1, Hut-78 and A-549 (Winarno, 2012). *P. macrocarpa* fruit extract has been used to treat C3H mice with breast cancer (Riwanto et al., 2011). Methanol extraction of *P. macrocarpa* fruits has anticancer activity against myeloma cell culture (Kurnijasanti et al., 2008).

P. macrocarpa fruit extract can reduce hyperglycemia in both *in vitro* and *in vivo* situations by effectively inhibiting carbohydrate-hydrolyzing enzymes. This finding proposes *P. macrocarpa* as a natural source for the management of type II diabetes mellitus (Ali et al., 2013). Pulp extraction of *P. macrocarpa* can stimulate the proliferation of B cells isolated from the Langerhans pancreas of the rat and decreases blood glucose levels (Arjadi & Susatyo, 2010).

Benzophenone glucoside was isolated from the *P. macrocarpa* bark ethyl acetate extract and was proven to have inhibitory activity against the leukemia L1210 cell line (Winarno & Ermin, 2010). A benzophenone aglucon 2,6,4'-trihydroxy-4-Methoxybenzophenon isolated from ethyl acetate extract of *P. macrocarpa* leaves showed strong antioxidant activity on DPPH (Susilawati et al., 2011; Tambunan & Simanjuntak, 2006).

Md Othman et al. investigated the cytotoxic potential of two benzophenone compound derivatives from ethyl acetate extract of *P. macrocarpa* fruits, identified as 2,6,4'-trihydroxy-4-methoxybenzophenone (compound 1) and 6,4'-dihydroxy-4-methoxybenzophenone-2-*O*- β -D-glucopyranoside (compound 2). The cytotoxic activities of these compounds were tested on the mouse embryonic fibroblast cell line (3T3)

and human cervical carcinoma cell line (HeLa). The results showed that benzophenone compound 1 revealed low cytotoxic effects against 3T3 cell and HeLa lines, while benzophenone compound 2 was nontoxic against 3T3 cell and HeLa lines (Md Othman et al., 2013). Gallic acid, a natural antioxidant was isolated from fruits of *P. macrocarpa* and its effect demonstrated on the inhibition of cell proliferation in cancer cell lines. The results showed that gallic acid could induce apoptosis in esophageal cancer cells (TE-2), promote apoptosis through up-regulating the pro-apoptosis BAX protein and inducing caspase-cascade activity in cancer cells (Faried et al., 2007).

Hendra et al., 2011b isolated and identified flavonoid compounds from *P. macrocarpa*. The results showed that myricetin, kaempferol, rutin and naringin were the main flavonoids existing in the pericarp, while quercetin and naringin existed in the mesocarp and seed. Furthermore, the study investigated the antibacterial activity of various parts of the *P. macrocarpa* fruit and showed a weak ability to moderate antibacterial potential against pathogenic tested bacteria, while antifungal activity was only present in the seed extract against *Aspergillus niger*. *P. macrocarpa* fruit could be considered a natural antimicrobial source because of the existence of flavonoid compounds. DLBS1425a compound isolated from an extract of *Phaleria macrocarpa* was investigated for its effect on the breast cancer cell line (MDA-MB-231). DLBS1425 revealed inhibition of proliferation, migration and expansion potential of MDA-MB231 by significantly encouraging proapoptosis genes BAX and Bad thus inducing cellular death signals by activating caspase-9 and promoting DNA fragmentation. These results suggest that DLBS1425 has potential as an anticancer agent (Tandrasasmita et al., 2010).

2.4.3.2 Ethnopharmacology of *Tinospora crispa*

The genus of the *Tinospora* plant has been known as a traditional medicine in South-east Asian countries. *Tinospora* has long been used in India as a medication and in the preparation of a starch known as *gilae-ka-sat* or as *palo*. It is a tonic, a diuretic and an antiperiodic. *Tinospora crispa*, which is abundant in the Philippines, is widely used by the population under the name of *makabuhany*, which means "You may live". It is used as an antidote and is especially valuable in general weakness, malarial fevers and chronic rheumatism. The whole plant contains the bitter principle colombine, alkaloids, flavonoids and glucoside (Dweck & Cavin, 2006).

A previous study was done to evaluate the nutritional components and mineral content of the stem of *T. crispa*. Results revealed that *T. crispa* contains a high moisture content of around 77.9%, carbohydrate content 19.4%, while the percentages of fat, protein, fiber and ash are low. The most abundant elements are calcium and potassium and other trace elements such as silicon, magnesium, chlorine and phosphorus are very low. The results also demonstrated that *T. crispa* extract has a high antioxidant property. This result was confirmed by the existence of phenolic and flavonoids in the extract as catechin, luteolin, morin and rutin, which are responsible for the high antioxidant activity. Outcomes from this study suggested that *T. crispa* could be an important source of nutrients and natural antioxidant (Amom et al., 2009). The variety in the chemical components of *T. crispa* make this plant useful in the treatment of different diseases and many studies have proved the benefits of *T. crispa* stem extract as an anti-parasite. *T. crispa* stem extract has been used *in vivo* as an antimalarial agent (Rungruang & Boonmars, 2009; Vigneron et al., 2005). *T. crispa* stem extract has been tested *in vitro* as an antifi-larial activity against adult worms of *Brugia malayi* (Zaridah et al., 2001). Isolated alkaloids from *T. crispa* stem fraction have been shown to possess *in vitro* anti-parasitic ac-

tivity against *Toxoplasma gondii* (Lee et al., 2012). This plant has been tested as an antinociceptive and an anti-inflammatory in various animal models (Sulaiman et al., 2008) and has been shown to have an inhibitory effect on edema formation in footpad injury that has been induced by carrageenan in rats (Higashino et al., 1992). The antioxidant properties of *T. crispa* stem extract could be used in the treatment of diseases resulting from the oxidative stress of cardiovascular disease (Ihsan et al., 2011). Various parts of the plant have been subjected to the study of antimicrobial activity. One study was performed to determine the *in vitro* antimicrobial ability of ethanol, methanol, distilled water and chloroform crude extract of *T. crispa* root. The results showed all the different extracts had antimicrobial activity against gram-negative bacterial strains of *Escherichia coli*, gram-positive bacterial strains of *Streptococcus pneumoniae*, and fungal strains of *Candida albicans* (Mohammed et al., 2012). Another study evaluated the biological activities of the stem bark *T. crispa* in different solvent extractions (methanol, petroleum ether, carbon tetrachloride, chloroform and aqueous). All extracts were subjected to antimicrobial screening against gram positive and gram-negative organisms and antioxidant screening by DPPH free radical scavenging activity. The results showed strong antioxidant activity, compared with ascorbic acid and butylated hydroxytoluene (BHT), as well as antibacterial activity (Aminul et al., 2011). Stem ethanol extract of *T. crispa* has an effect against Methicillin-resistant *Staphylococcus aureus* (Al-alusi et al., 2010). *T. crispa* has been traditionally known as an anticancer drug, thus many studies have subjected this plant to investigations of anticancer activity. *T. crispa* has a dose-dependent anti-proliferative activity against cancer cells MDA-MB-231, HeLa and human mammary cancer cells (MCF-7) (Ibrahim et al., 2011). The aqueous crude extract of *T. crispa* stem has anti-proliferative activity against the MCF-7 cell line (Amom et al., 2008). The influences and mechanisms of the *T. crispa* extract on blood pressure and a heart

disease has been thoroughly studied. Crude extract and active compounds (salsolinol, higenamine, tyramine, adenosine and uridine) of *T. crispa* stem show a positive inotropic impact on the electrical field. It excited the isolated left atria by affecting either the adrenergic receptors to increase the force of the atrial contraction or directly through encouraging the release of acetylcholine and the purinergic pathways causing negative inotropic effectiveness on the isolated left atria (Praman et al., 2013).

Kongkathip et al. isolated two compound triterpenes from the stems of *T. crispa*, cycloeucalenol and cycloeucalenone, and investigated their effect on cardiac contractility. They found that cycloeucalenol increased the right atrial force of contraction, whereas it showed a preliminary decrease followed by a continued reduction of about 10% in the left atria of the rat *in vitro*. Cycloeucalenone exhibited a small alteration in the control of the right and left atrial force. These results propose that cycloeucalenol and cycloeucalenone create slight cardiotonic effects (Kongkathip et al., 2002). N-butanol extract from the stems of *T. crispa* extract was subjected to a study on its effectiveness on blood pressure and heart rate in rats, and then the four compounds in the extract (epinephrine, tyramine, hordenine, and pseudoephedrine) were identified using the HPLC method. These results suggest that *T. crispa* extract has at least three different cardiovascular active components that work directly via B₂ – adrenergic receptors to decrease blood pressure (Praman et al., 2011). *T. crispa* stem aqueous extract supplementation in a hypercholesterolemia-induced rabbit showed a reduction in triglycerides, total cholesterol and low-density lipoprotein levels, besides decreasing the value of the atherosclerotic index in a *T. crispa* treated group compared to the hypercholesterolemia control group. *T. crispa* can be included as one of the therapeutic agents that can be a hypocholesterolemic drug and prevent atherosclerosis (Amom et al., 2011).

T. crispera aqueous and methanol extracts prevent the oxidative stress and inflammation induced by hydrogen peroxide and TNF- α that causes damage to the human umbilical vein endothelial cells, through its effect on inducing antioxidant enzymes CAT, SOD and GPx activity (Kamarazaman et al., 2012).

In Malaysia, an aqueous extract of *T. crispera* stems is taken orally for treatment of diabetes mellitus. A study was done using normal and alloxan diabetic rats to evaluate the hypoglycemic potential of the extract. After two weeks treatment with the extract there was an improvement in glucose levels. Furthermore, acute intravenous treatment with the extract caused an increase in plasma insulin levels. These findings support the traditional belief that *T. crispera* extract could improve diabetic conditions by improving pancreendocrine function (Noor & Ashcroft, 1989). Many previous studies have shown that the antihyperglycemic properties of *T. crispera* have an effect on intestinal glucose absorption and glucose uptake into adipocytes (Noor & Ashcroft, 1998). The effect of *T. crispera* on serum glucose and insulin levels in healthy individuals and patients with type II diabetes mellitus has been investigated (Klangjareonchai & Roongpisuthipong, 2011). Other studies investigated the effectiveness of *T. crispera* aqueous extract on glucose transport action in the skeletal muscle cell line. The results showed that *T. crispera* aqueous extract enhances glucose transport through an insulin independent pathway in a time and dose dependent manner (Noipha et al., 2010). Ruan et al. explored hypoglycemic activity in mice for active compounds, borapetosides A, B and C, isolated from the ethanol extract of the *T. crispera* stem. The results showed hypoglycemic effects through both the insulin dependent and the insulin independent pathways with an increase in the glucose consumption in peripheral tissue, reducing hepatic gluconeogenesis and stimulating the insulin-signaling pathway (Ruan et al., 2012; Ruan et al., 2013).

CHAPTER THREE

3 METHODS

3.1 Plants collection and extraction

3.1.1 Plant collection

Phaleria microcarapa are dried seedless fruits and *Tinospora crispa* are dried stems collected from the Bandar Baru Bangi, Selangor housing area. The two plants were authenticated by Mr. Shamsul Khamis, a botanist at the Institute of Bioscience (IBS), University Putra Malaysia (UPM). A voucher specimen (SK1929/11) for *Phaleria microcarapa* and (KLU 45568) for *Tinospora crispa* were preserved in the herbarium of IBS, UPM.

3.1.2 Plant extraction

3.1.2.1 A solid-liquid extraction

3.1.2.1.1 Theory of test

In the simplest form of this operation, the extraction material is mixed with an adequate amount of solvent to allow the solvent to immiscible completely with the extracted material to ensure the the total penetration of the solvent and its ability to dissolve the desired components. The solvent and the dissolved transition material were then separated from the raw material and the solvent evaporated.

3.1.2.1.2 General procedure

The dried plant was crushed and the powder (100 g) was placed in a conical flask and soaked in 900 ml 95% of ethanol for three days at room temperature (30 ± 2 °C). The suspension was shaken from time to time to allow the powder to dissolve completely in the ethanol and the color to change to dark brown. The mix was filtered after three days using a filter paper (Whitman, 185 mm) and the extract was distilled under reduced pressure in a rotary evaporator (Buchi, Switzerland). The extract was maintained at -20 °C prior to usage (Trusheva et al., 2007).

3.2 Antioxidant and free radical study

3.2.1 Ferric reducing antioxidant power (FRAP)

3.2.1.1 Theory of test

The FRAP assay has been used to assess total antioxidant ability in biological fluids, such as plasma and plant extract samples. The method is based on the antioxidant ability of the sample as a reductant to reduce oxidants present in the reagent. Through reduction-oxidation reaction (redox), the colorimetric assay is linked to react with a ferritripyridyl triazine (FeIII-TPTZ) complex at low pH and produces a blue colored ferrous tripyridyl triazine (FeII-TPTZ). FeII – TPTZ has an intensive blue color and is detected spectrophotometrically at 593nm (Griffina & Bhagooli, 2004).

3.2.1.2 General procedure

This method was done by preparing the working FRAP reagent as described below:

- 1) The 300 mmol/L acetate (pH 3.6), buffer was prepared by mixing 3.1g of sodium acetate trihydrate ($C_2H_3NaO_2 \cdot 3H_2O$) with 16ml glacial acetic acid/ L of buffer solution.

2) The TPTZ (2,4,6-tripyridyl-s-triazine) solution was prepared by making a solution of 10 mmol/L TPTZ in 40 mmol/L HCL.

3) 20 mmol/L $\text{FeCl}_3 \times 6 \text{H}_2\text{O}$ was added to distilled water.

4) The three above reagents was mixed in a percentage of 10:1:1 respectively, by adding 25ml acetate buffer, 2.5ml TPTZ solution and 2.5ml $\text{FeCl}_3 \times 6 \text{H}_2\text{O}$ solution. The working solution was freshly prepared.

5) A prepared standard of known Fe (II) concentrations ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) run in triplicate was used for calibration in several concentrations between (100-1000 $\mu\text{mol/L}$).

6) Usage of FRAP reagent as blank.

150 μl of total FRAP reagent was added to each well in a 96-well (300 μl) micro-plate, 20 μl of sample was then added to each well in triplicate, then after 15 min the absorbance was measured at 593 nm. The relative activities of samples were assessed by comparing their activities with that of a Fe^{+2} equivalent (Griffina & Bhagooli, 2004).

3.2.1.3 Calculations

A standard curve was then plotted (standard concentration versus the average FRAP value for each standard concentration). The FRAP values for the samples was then determined using this standard curve.

3.2.2 Redical scavenging activity test (DPPH)

3.2.2.1 Theory of the test

DPPH is an easy and inexpensive method used to determine the antioxidant activity of foods, plants and compounds. In addition, it is used to quantify antioxidants in a complex biological system. The liquid and solid samples can detect their antioxidant ability using this method. Finally, the DPPH method is nonspecific for any individual antioxidant compound but can be used for any sample which has antioxidant ability.

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable free radical that can donate hydrogen atoms and can be scavenged by any antioxidant compound. When it is dissolved in solvent, it gives a purple color. The color changes to yellow when a single electron of DPPH radical is doubled with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH form. The color is become more decolorization that indicates an increase in the number of electrons captured (Generalic et al., 2011).

3.2.2.2 General procedure

The test was done according to the method described by (Chun et al., 2005; Generalic et al., 2011). One mg of plant extract was dissolved in 1ml ethanol 95% to make a stock solution and then five dilutions were made from the stock. A standard L-ascorbic acid was prepared in the same way as the plant extract. Five μ l from each dilution of plant extract and standard were added to 195 μ l DPPH and then loaded in the 96-well plate in triplicate for each sample. Ethanol was used as the blank. The plate was incubated at room temperature for 2hrs and the absorbance was read at 517nm.

3.2.2.3 Calculation

The antioxidant activity was calculated as DPPH radical scavenging activity (DPPH inhibition %) according to the following:

$$\text{DPPH inhibition \%} = [(\text{absorbance blank} - \text{absorbance sample}) / \text{absorbance blank}] \times 100$$

A graph was plotted according to the concentration of sample versus the DPPH scavenging activity percentage to evaluate the IC₅₀ (the concentration needed to inhibit 50% of DPPH radical scavenging activity (Luís et al., 2009; Scherer & Godoy, 2009)).

3.2.3 Total phenolic content

3.2.3.1 Theory of the test

The Folin-Ciocalteu method was used to determine the total phenolic content in the *P. macrocarpa* and *T. crispera* extract. The test was based on the oxidation-reduction reaction that happens between the Folin-Ciocalteu reagent solution (phosphomolybdic and phosphotungstic acid) and the phenolic compounds in the sample. The reagent oxidizes ionized phenolic groups that exist in the sample and reduces the acid to form a blue complex. The blue color is developed in an alkaline medium reaction that is supplied by sodium carbonate, and the measurement of the blue color optical density is done at 765 nm (Reynaud et al., 2010).

3.2.3.2 General procedure

Total phenolic content is determined as described by (Kubola & Siriamornpun, 2008; Reynaud et al., 2010). 10 µl from extracts prepared at a concentration of 1 mg/ml was added to 100 µl of Folin-Ciocalteu reagent (prepared earlier in a 10-fold dilution with deionized water) then mixed well and left for 5 min at room temperature. After that, 100 µl from 10% sodium carbonate was added, the mixture was incubated for 2 h at room temperature and then the absorbance of optical density was read at 765 nm. Gallic acid was used as the standard.

3.2.3.3 Calculation

The standard calibration (0-200 mg/ml) curve was plotted using gallic acid, and the total phenolic content was expressed as gallic acid equivalent in mg/ g of dry sample or mg GAE/ g sample.

3.2.4 Estimation of total flavonoids content

3.2.4.1 Theory of the test

Flavonoids are polyphenol compounds soluble in water, which are common and largely spread in plants as a product of the glycoside process. An aluminum chloride colorimetric assay is used for the determination of total flavonoids. The test is performed by the reaction between aluminum chloride and the C4 keto group and either C3 or C5 hydroxyl group of the flavonoids forms a stable acid complex (Rajanandh & Kavitha, 2010).

3.2.4.2 General procedure

The aluminum chloride colorimetric method was used in determining flavonoids performed as described by (Ghasemia et al., 2009; Pourmorad et al., 2006; Ramamoorthy & Bono, 2007). Briefly, the method was fulfilled by mixing 0.5 ml of plant extract (1mg/ml), 1.5 ml of methanol, 0.1ml of 10% aluminum chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water. The mixture was then left at room temperature for 30 minutes, and after that the absorbance reaction was read at 415 nm.

3.2.4.3 Calculation

A calibration curve was done to calculate total flavonoids contents. The calibration curve was done by preparing a concentration of 0 – 200 mg /ml from quercetin solution in methanol. The total flavonoids content was expressed as a quercetin equivalent in mg/g of dry sample or mg QE/g sample.

3.3 Acute toxicity test

3.3.1 Theory of the test

Groups of animals of both sexes are administrated a single dose by the oral route. The dose level is chosen on the basis of an expected dose that will produce some toxic signs without causing severe toxicity signs or leading to mortality. Any clinical signs that are seen or any death would be recorded. The safety of the dose level depends on the results, the level of toxicity that occurs and death if it happens (OECD, 2001).

3.3.2 Animals

Adult Sprague Dawley rats, both male and female (6-8 weeks old) and weighing between 160 g to 180 g, were used. These rats were obtained from the Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur (Ethics No. PM/07/05/2008/1111MAA (a) (R) in01/November 2011. All rats were housed individually and maintained under standard conditions of humidity (50-60%), temperature ($22 \pm 3^{\circ}\text{C}$) and light (12h light: dark cycle) and were fed purina chow diet and water ad libitum. For the experiment, each rat was caged alone and fasted prior to dosing (food, but not water, was withheld overnight).

3.3.3 Acute toxicity test

There were four groups of 12 adult Sprague Dawley (SD) rats, each group comprising six males and six females. Each rat was caged alone and fasted prior to dosing (food, but not water was withheld overnight). The plant extracts were dissolved in Tween 20 (5% v/v) and administered at single doses of 50, 500 and 2000 mg/kg weight per rat (5 ml/kg). The control group received the same volume of Tween 20 (5% v/v). Both treatment and control groups were administered single doses orally via an oral gavage tube. After the plant extract was administered, food was withheld for 3 h to 4 h.

Toxicity symptoms and mortality were observed after dosing once during the first 30 min and recurrently during the first 24h with special concentration on the first 4 h and then daily for a total of 14 days. After the 14th day, the animals from each group were anesthetized with 0.01 ml/kg xylazine and 0.09 ml/kg ketamine and then sacrificed. Blood, liver, and kidney samples were collected for histopathology and clinical biochemistry examinations. A toxicity study was performed as per Organization for Economic Co-operation and Development guideline 420 (OECD, 2001).

3.4 Gastroprotective ability of the plant extract

3.4.1 Animals

Healthy Sprague Dawley female rats weighing between (200 g to 250 g) were used. The animals were randomly divided into seven groups (six rats/ group) and were obtained from the Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur. The experimental protocol was approved by the animal ethics committee (Ethics No. PM28/09/2006/1111MAA (a) (R) in 1/November 2011. All rats were housed individually and maintained under standard conditions of humidity (50-60%), temperature ($22 \pm 3^{\circ}\text{C}$) and light (12h light: dark cycle) and fed purina chow diet and water ad libitum. For the experiment, each rat was caged alone and fasted before treating (food withdrawn overnight). Throughout the experiment, all criteria for taking care of animals provided via the National Academy of Sciences and defined in the “guide for the care and use of laboratory animals” were applied (Clark et al., 1997).

3.4.2 Experimental design

Dry extract was dissolved in Tween 20 (5% v/v) and was given orally to rats in dosages of 100, 200, 500 and 1000 mg/kg body weight for each plant. Ethanol 95% at (5ml/kg) was administered orally to induce gastric ulcer in all groups except the vehicle

control group. Omeprazole 20 mg/kg was dissolved in Tween 20 (5% v/v) and the solution was orally administered to rats (5ml/kg) body weight (Table 3.1). Water was removed 1h before the experiment. For the first group (vehicle control group), the rats were administered vehicle Tween 20 (5% v/v) (5 ml/kg). The second group (ulcer control group), the rats received Tween 20 (5% v/v) (5 ml/kg) and for the third group (reference drug group), the rats were pretreated with omeprazole solution. For the fourth group, the rats were pretreated with 100, 200, 500 and 1000 mg/kg plant extracts. After 1 h, all rats received 5 ml/kg 95% ethanol orally to induce gastric ulcer (except for the first group) and after 1h, the rats were killed and blood and stomach were collected from each rat (Abdulla et al., 2010; Salga et al., 2012).

Table 3.1: Gastroprotective activity of plant extracts experimental design.

Group	Name of group	Orally administration	Time	Orally administration
1	Vehicle control	5ml/kg Tween 20	After one hour	5ml/kg Tween 20
2	Ulcer control	5ml/kg Tween 20	After one hour	5ml/kg 98% ethanol
3	Reference drug	20 mg/kg solution (5ml/kg)	After one hour	5ml/kg 98% ethanol
4	Pretreated group 100mg/kg	100mg/kg solution (5ml/kg)	After one hour	5ml/kg 98% ethanol
5	Pretreated group 200mg/kg	200mg/kg solution (5ml/kg)	After one hour	5ml/kg 98% ethanol
6	Pretreated group 500mg/kg	500mg/kg solution (5ml/kg)	After one hour	5ml/kg 98% ethanol
7	Pretreated group 1000mg/kg	1000mg/kg solution (5ml/kg)	After one hour	5ml/kg 98% ethanol

3.4.3 Measurement of ulcer index

The ulcers were located in the gastric mucosa and appeared as a hemorrhagic extended band of wounds parallel to the extended axis of the stomach. The gastric mucosa ulcer was calculated by measuring the sum of all lesion areas for each stomach and calculating the ulcer area (UA) equal to the sum of small squares $\times 4 \times 1.8 = \text{UA mm}^2$. The ulcer inhibition percentage (UI %) was calculated by (Ketuly et al., 2011).

$$\text{UI \%} = [(\text{UA control} - \text{UA treated}) / \text{UA control}] \times 100\%.$$

3.4.4 Measurement of gastric juice pH

After the stomach was opened, the gastric juice was collected and measured for gastric juice pH using a digital pH meter (Barros et al., 2007).

3.4.5 Histology estimation of gastric lesions

A piece of the glandular portion of the stomach was fixed in 10% formalin and then dehydrated by alcohol using ascending grades in the automated tissue processing machine (Leica Microsystems, Nussloch, Germany) (appendix I). The processed tissues were embedded in paraffin to prepare for sectioning and a microtome (Leica Microsystems, Nussloch, Germany) was used to cut the tissues in 5 μm sections for hematoxylin–eosin staining according to standard protocols (appendix I) (Suzuki et al., 2012).

3.4.6 Tissue homogenate sample preparations for assessment of prostaglandin E 2 (PGE2), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA)

To assess PGE2 (Cayman PGE2 assay ELISA kit; Cat# 400144), SOD (Cayman, Cat#. 706002), CAT (Cayman Cat#. 707002) and MDA (Cayman Cat#. 10009055) in gastric tissue homogenate, the gastric tissue was weighed, minced and homogenized on ice in 5 ml cold PBS buffer using a Teflon homogenizer (Polytron, Heidolph RZR 1, Germany). After each piece of stomach tissue was fully homogenized, the mixture was centrifuged at 10.000xg for 15 min at 4 °C. Then the supernatant was collected in a sterile tube and kept at (−80 °C) until use. The concentration of protein was performed by the Bradford method using bovine serum albumin as a standard (Barford, 1976). The assays were performed as per the detailed instructions of the manufacturer (appendix II). The sensitivity limit of these assays was ≤ 15.6 pg for PGE2.

3.4.7 Preparation of blood sample for assessment of serum gastrin, pepsin, TGF- β 1 and TNF- α

After the rat blood samples were collected in tubes, they were allowed to clot for 30 min at 25 °C. Then they were centrifuged at 2000xg for 15 min at 4 °C using a refrigerated centrifuge Rotofix 32 (Hettich Zentrifugen, Germany). The serum was collected and preserved at −80 °C until use. Gastrin was measured by an Abnova rat gastrin ELISA kit (Cat#. KA0319 V.01), pepsin by a Cusabio rat pepsin assay ELISA kit (Cat#. CSB-E 08920r). TGF- β 1 by an ELISA kit (Abnova, Cat#. KA0279; version 4) and rat TNF- α by an ELISA kit (Thermo Scientific, Cat#. ER3TNFA). The sensitivity limit of these assays were 7.8 pg/ml for TGF- β 1, <15 pg/ml for TNF- α , ≤ 78.10 pg for gastrin and ≤ 0.8 ng for pepsin. The assays were performed as per the detailed instructions of the manufacturer (appendix II).

3.5 Determination of gastric wall mucus content

The gastric mucus contents were determined according to the assay procedures previously described (Freitas et al., 2011). Adult Sprague Dawley female rats weighing between 200g to 250g were fasted for 24h. The animals were randomly divided into four groups (six rats per group) and water was removed 1h before the experiment. For the first group (vehicle control group), the rats were given vehicle Tween 20 (5% v/v) (5 ml/kg). The second group (ulcer control group) received Tween 20 (5% v/v) (5 ml/kg). The third group (reference drug group) was pretreated with 20 mg/kg omeprazole solution (5 ml/kg) and the fourth group was pretreated with 250 mg/kg *T. crispa* stem extract. For the fifth group, the rats were pretreated with 250mg/kg *P.macrocarya* extract. After 1 h of pretreatment, all the rats received 5 ml/kg of 95% ethanol orally to induce gastric ulcer (except for the first group). After 1h, all the rats were sacrificed by euthanasia and their stomachs were removed. Each glandular part was weighed and submerged into 1% Alcian blue solution (0.16 M sucrose/ 0.05 M sodium acetate, pH 5.8). After 2h of immersion, the excess stain was rinsed with 10 ml sucrose at 0.25 M for two consecutive washes: the first time for 15 min and the second for 45min. Then the stomachs were transmitted to tubes containing 10 ml magnesium chloride at 0.5 M for 30 min. After 30 min, 4 ml of the mixture was mixed with 4 ml of ethyl ether and then shaken for 2 min. The final emulsion was centrifuged for 10 min at 3000 rpm and then the supernatant was discarded. The absorbance was read at 598nm and the calculation of the amount of Alcian blue was taken as per gram of glandular tissue.

3.6 Wound healing ability of plant extract

3.6.1 Animal

The animals used were healthy adult Sprague Dawley female rats weighing 250 gram to 300 gram and the rats were obtained from the Animal House, Faculty of Medicine, University of Malaya, Kuala Lumpur. The experimental protocol was approved by the animal ethics committee (Ethics No. PM28/09/2006/1111MAA (a) (R) on 1/November 2011. All rats were housed individually and maintained under standard conditions of humidity (50-60%), temperature ($22 \pm 3^{\circ}\text{C}$) and light (12h light: dark cycle) and fed purina chow diet and water ad libitum. For the experiment, each rat was caged alone and fasted before treatment (food, but not water, was withdrawn overnight). Throughout the experiments, all criteria for taking care of animals provided by the National Academy of Sciences and defined in the “guide for the care and use of laboratory animals” are applied (Clark et al., 1997).

3.6.2 Experimental design

The vehicle used during the study was gum acacia dissolved in normal saline with ethanol extract. Two g of gum acacia was dissolved in (100 ml) of normal saline. Ten ml of solution was used to dissolve 1 g and 2 g of ethanol extract and each ml of solution contained 100 mg or 200 mg of extract (Shetty et al., 2008). The adult SD rats were randomly divided into six groups, with five rats in each group (Table 3.2). Each rat was housed separately. When the animals were anesthetized, their skins were shaved using an electrical shaver and they were disinfected with 70% alcohol. A uniform wound area 2.00 cm in diameter was excised from the nape of the neck of all the rats using a round seal, as described by Morton et al. (Morton & Malone, 1972). Incision of the muscle layer was avoided and skin tension was kept constant during the procedure.

All of the rats were treated twice a day. As mentioned below:

- The vehicle control group with 0.2 ml gum acacia.
- The reference drug group with 0.2 ml Intrasisite gel.
- One of the treated groups with 0.2 ml of 100 mg/ml plant extract.
- The other treated group with 0.2 ml of 200 mg/ml plant extract.

The contraction of the wound area was measured on 0 day, 5th day, 10th day, and 15th day. On the 15th day, all of the rats were administered high doses of anesthesia and the skin from the healed wound area was excised to obtain homogenate tissue for histopathological examination. Blood samples were also obtained to measure other parameters.

Table 3.2: Wound healing activity of plant extracts experimental design.

Group	Name of group	Treated
1	Vehicle control	Gum acacia solution
2	Intrasisite gel	Topical Intrasisite gel
3	Low dose of extract	100mg/kg
4	High dose of extract	200mg/kg

3.6.3 Histological evaluation of wound healing

A piece of skin from the healed wound area was fixed in 10 % formalin and then dehydrated by alcohol in ascending grades in an automated tissue processing machine (Leica Microsystems, Nussloch, Germany). The processed tissue was embedded in paraffin to prepare the tissue for sectioning. A microtome (Leica Microsystems, Nussloch, Germany) was used to cut the tissue into sections of 5 μm to prepare for staining with hematoxylin–eosin solutions according to standard protocol (appendix I) (Suzuki et al., 2012).

3.6.4 Homogenate tissue sample preparations for superoxide dismutase (SOD), catalase (CAT), and MDA assessment

To assess SOD (Item No. 706002), CAT (Item No. 707002), and (MDA) (Item No. 10009055) in the homogenate skin tissue, the skin tissue was weighed, minced, and homogenized on ice in 5 ml cold PBS buffer using a Teflon homogenizer (Polytron, Heidolph RZR 1, Germany). After each piece of skin tissue was fully homogenized, the mixture was centrifuged (refrigerated Rotofix 32 centrifuge, Hettich Zentrifugen, Germany) at 10.000xg for 15 min at 4°C. Then the supernatant was collected in a sterile tube and kept at (–80 °C) until use. Concentration of protein was performed by the Bradford method using bovine serum albumin as a standard (Barford, 1976). The assays were performed as per the detailed instructions of the manufacturer (appendix II).

3.6.5 Measurement of TNF- α and TGF- β 1 by enzyme – linked immunosorbent assay (ELISA)

The blood samples of the rats were collected in tubes. The blood was allowed to clot for 30 min at 25°C, then centrifuged at 2000xg for 15min at 4 °C using refrigerated centrifuge Rotofix 32 (Hettich Zentrifugen, Germany) then the serum was collected and preserved at -80°C until use. The measurement of rat TGF- β 1 was by ELISA kit (Abnova, Cat#. KA0279; version: 04) and rat TNF- α by ELISA kit (Thermo Scientific, Cat#. ER3TNFA). The assays were performed as per the detailed instructions of the manufacturer (appendix II). The sensitivity limit of these assays was TGF- β 1 is 7.8 pg/ml and <15pg/ml for TNF- α .

3.7 BAX and BCL2 gene expression *in vivo* (wound healing model)

The skin samples from the healed wound (the area in the section of the wound healing experiment) were kept in an RNA solution (Ambion, Austin, Texas, USA) and preserved at 4°C overnight , then kept at -80°C until RNA extraction was complete.

3.7.1 RNA extraction

Total cellular RNA was extracted and purified using a commercialized Gene JET RNA purification kit (Thermo Scientific, Cat. No: K0731). The extraction and purification processes were performed as per the instruction protocol. 30 mg of frozen tissue was weighed, pounded using a mortar and pestle, then 600 μ l of diluted proteinase K (10 μ l of the concentrated proteinase K diluted in 590 μ l of TE buffer) was added. The mixture was vortexed, incubated at 15-25°C for 10 min, then centrifuged for 10min at 12000xg. The supernatant was transferred into an RNase free microcentrifuge tube. After that 450 μ l of 96-100% ethanol was added to the mix, then 700 μ l of lysate was transferred to the gene JET RNA purification column, centrifuged at 1200xg for 1 min. The

purification column was placed in a new collection tube and 700 µl of buffer1 (supplemented with kit) was added, before centrifuging at 1200xg for 1min. To eliminate genomic DNA contamination the mixture was treated with 80µl DNaseI (Qiagen, Hilden, Germany, Cat.No:79254). The purification column was placed into a new collection tube and 600µl of wash buffer2 was added (supplemented with kit). The previous step was repeated with 250µl of wash buffer2. Finally, the purification column was placed in the nuclease free 1.5 collection tube to elute the RNA by adding 100µl of nuclease free water and centrifuged for 1min at 1200xg. The purified RNA was kept at -80°C until use.

The RNA concentration and integrity were electrophoretically verified by optical density ($A_{260}/A_{280} \geq 1.65$) measured by a Nano Drop ND- 2000 spectrophotometer (Thermo Fisher scientific, Wilmington, DE, USA), and by ethidium bromide staining with 2 % denaturing agarose gel electrophoresis (Bio-Rad, Richmond, CA, USA) and ethidium bromide staining (appendix III) (Pfaffl et al., 2003) .

3.7.2 Reverse transcription (RT)

The total RNA extracted was used for cDNA synthesis. Reverse transcription was performed using a commercialized kit (high capacity RNA to cDNA kit, applied Biosystems, Cat.No.4387406). Two µg of total RNA were used /20 µl reaction as instructed in the user's protocol. For every 20µl reaction, the total RNA sample was mixed with an equal volume of the 20X Enzyme mix as detailed in (Table 3.3). The RT reaction was aliquoted in the tube and the cap was sealed and briefly centrifuged to eliminate any air bubbles. The reaction was then incubated in the thermal cycler (MJ Research PTC-100 Thermal cycler, USA) at 37°C for 60 min. The reaction was stopped at 95°C for 5 min and was held at 4°C, the cDNA was then stored at (-15°C to -25°C) until used in real time PCR.

Table 3.3: Composition of the reverse transcriptase reaction

Component	Component volume/ reaction (μl)
2X RT buffer	10.0
20X Enzyme mix	1.0
RNA sample	Up to 9 μl
Nuclease-free water	Quantity sufficient to 20 μl
Total per reaction	20

3.7.3 Real time reverse transcriptase polymerase chain reaction (RT-PCR) gene expression assay

3.7.3.1 TaqMan chemistry

The effect of the plant extract on the BAX and BCL2 gene expression was determined using a real time PCR assay. Reverse transcription PCR based assays are currently the most common method for characterizing and confirming gene expression levels from different sample populations. Real time systems for PCR have been improved by being probe based. The real time PCR technique as it is used today depends on two important principles. First, the TaqMan polymerase plays an important role in the (5'–3' exonuclease activity) polymerase activity. Second, typical amplification reactions include the probe; it is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. Design and synthesis of the probe has been simplified by labeling with a reporter fluorochrome (e.g., 6-carboxyfluorescein, or FAM) at the 5' end and quenching (e.g.: 6-carboxy-tetramethyl-rhodamine or TAMRA) at the 3' end of the probe (Giulietti et al., 2001). The real time method is used to detect only specific amplification products. During the amplification and annealing of the probe to its target, the sequence generates a substrate that is cleaved by the 5' nuclease activity of TaqMan

DNA polymerase when the enzyme extends from an upstream primer into the region of the probe (Walker, 2002). This dependence on polymerization ensures that the cleavage of the probe occurs only if the target sequence is being amplified (Figure 3.1). The probe has a melting temperature (T_m) approximately 10°C higher than the T_m of the primers in order to anneal to the amplicon during the extension phase of the PCR process (which is performed at 60°C). Consequently (Overbergh et al., 2003), the probe will be degraded during the phase by the 5'-3' exonuclease activity of the TaqMan polymerase. This will result in an increase in reporter fluorescence emission because reporter and quencher are separated. The amount of fluorescence released is directly proportional to the amount of product generated in each PCR cycle and thus can be applied as a quantitative measure of PCR product formation (Bustin, 2000).

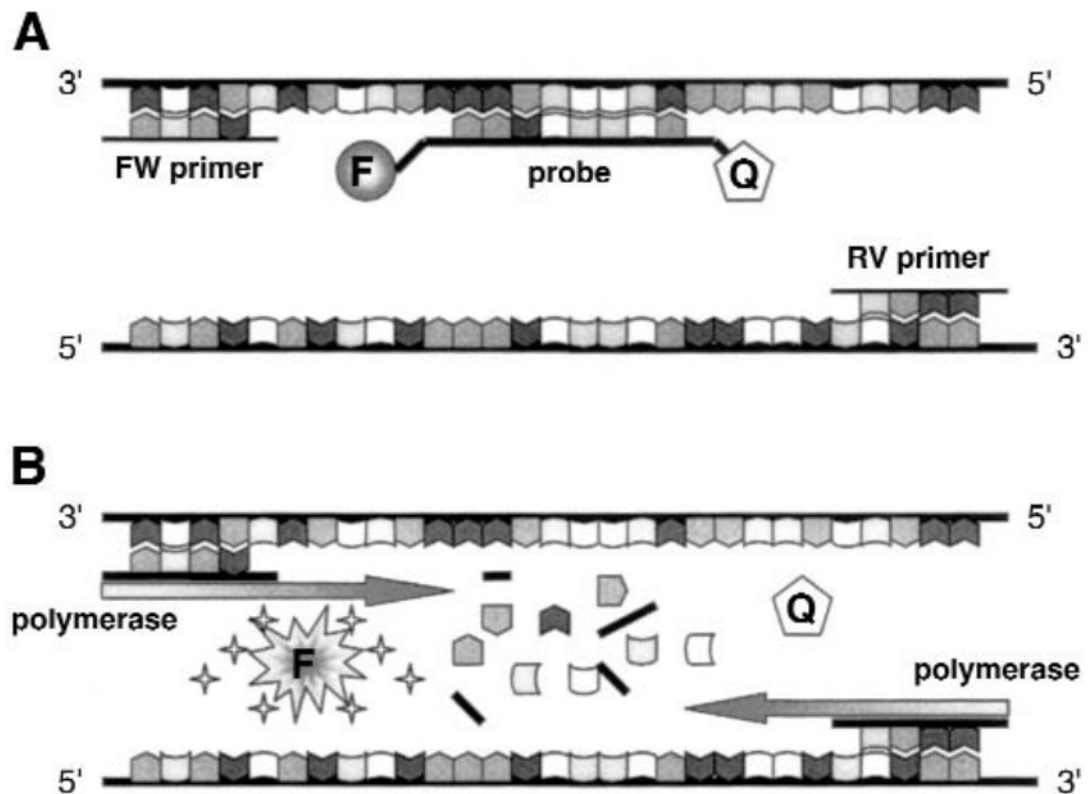


Figure 3.1: Schematic representation of the TaqMan principle. A: Primers and probe anneal to the target gene. Fluorescence emission does not occur because the probe is still intact. B: During the extension phase of the PCR reaction, the probe is cleaved by the 5′–3′ exonuclease activity of the TaqMan polymerase, allowing fluorescence emission. FW, forward; RV, reverse; F, fluorophore; Q, quencher dye. cited by (Overbergh et al., 2003).

3.7.3.2 Real time amplification assay

The reaction setup for all TaqMan Gene Expression assays was performed according to the manufacturer’s instructions for the TaqMan fast advanced master mix (Applied Biosystems Cat. No: 4444557) and generated by the Step One plus system software (Ver. 2.0, Applied Biosystems). Briefly the reaction was performed by mixing 5 µl of (2 X) TaqMan fast advanced masters with 0.5 µl of (20X) TaqMan Gene (Table 3.4) and 1 µl of cDNA; the reaction volume was adjusted to 10 µl with Nuclease-free water. All the above components were mixed in a 1.5 ml microcentrifuge tube, briefly vortexed to mix and centrifuged to eliminate the air bubbles and spin down the contents After that the appropriate volume was loaded in duplicate into the optical reaction plate well (Ap-

plied Biosystems MicroAmp fast optical 96-well reaction plate with barcode Cat. No: 4346906), the plate was completely covered with an optical adhesive cover (Applied Biosystems MicroAmp optical adhesive film, Cat. No. 4360954), and briefly centrifuged to eliminate the air bubbles and spin down the contents. Finally, the PCR reaction was run using Thermal cycling conditions as described in (Table 3.5).

Table 3.4: TaqMan Gene investigated

NO.	Gene name and abbreviation	TaqMan Gene Assay ID	NCBI Reference Sequence
1	BCI2-associated X protein (Bax)	Rn02532082_g1	NM_017059.1
2	B cell lymphoma -2 (BCL2)	Rn99999125_m1	NM_016993.1
3	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous control	Rn0177563_g1	NM_017008.3
4	Hypoxanthine phosphoribosyl transferase 1 (HPRT1) endogenous control	Rn01527840_m1	NM_012583.2

Table 3.5: thermal cycle conditions

Thermal cycling profile				
parameters	incubation	Polymerase activation	PCR (40 cycle)	
	Hold	Hold	Denature	Anneal/extend
Temperature(°C)	50	95	95	60
Time (mm:ss)	02:00	00:20	00:01	00:20

3.7.3.3 RT-PCR Analysis

Amplification efficiency in this experiment was performed to ensure that the amplification efficiencies of the target gene and endogenous gene were approximately equal. The slope of a standard curve is generally used to estimate the PCR amplification efficiency of a real time PCR reaction. A real time PCR standard curve is graphically represented as a semi-log regression line plot of C_T value versus log of input nucleic acid. A standard curve slope of -3.32 indicates a PCR reaction with 100% efficiency (Liss, 2002).

The comparative C_T method ($\Delta\Delta C_T$) expression of the investigated genes was normalized with the endogenous control (HPRT1). C_T values are means of duplicate measurements. Comparative C_T method quantification was determined by the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001). For statistical analysis of real time RP-PCR experiments, results for a given gene were expressed as difference from the ΔC_T value obtained between treated versus un-treated (Calibrator).

The calculation of fold change was done as described below:

$$C_T \text{ Target} - C_T \text{ Endogenous control} = \Delta C_T$$

$$\Delta C_T \text{ Sample} - \Delta C_T \text{ Calibrator} = \Delta\Delta C_T$$

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

3.8 Profiling and fractionation of crude extracts

One gram of ethanol crude extract was dissolved in 5 ml of methanol and subjugated to column chromatography fractionation in glass columns at 3.0 x 50 cm (Kontes Scientific Glassware, Vineland, NJ, USA) packed with silica gel G60, 70 - 230 mesh (Merck, Darmstadt, Germany) and linked with an EYEL-L4 pump (Tokyo Rikakikai, Tokyo, Japan). A gradient concentration of solvent that differed in polarity (25 ml each time of five different concentrations; 20, 40, 60, 80 and 100 %) was used to elute the crude extract. The solvents used were hexane, ethyl acetate, methanol, acetonitrile and water in order to increase the polarity, starting with lower polarity and ending with high polar solvent. The yield fractions were collected in a clean glass tube and gathered based on the similarity of the solvents used. Solvents were evaporated from yield fraction under reduced pressure using a centrifuge evaporator. The crude extract and all fractions were tested for their effect *in vitro* on the RAW 264.7 cell line. The fraction will affect RAW 264.7 cell proliferation and cause an immunomodulatory effect. It was subjected to liquid chromatography mass spectrometry (LC-MS) to determine the active constituents.

3.9 Immunomodulatory effect *in vitro*

3.9.1 Cell line

A murine macrophage cell line RAW 264.7 (Figure 3.2) obtained from the American Type Culture collection (ATCC, Rockville, MD) was cultured in DMEM and supplemented with 4500g glucose/L, 110mg sodium pyruvate/L and 1% penicillin-streptomycin (Sigma-Aldrich, UK) and 10% heated-in activated, endotoxin level less than < 0.1 EU/ml fetal bovine serum (FBS) (BIOWEST, France). The cells were cultured at 37°C in a 5% CO₂ atmosphere incubator (NuAire, Plymouth, MN, USA). For the experiments, the cells were sub cultured when they reached 80% confluence, then

the cell suspension was diluted to 5×10^5 cell/ml (Guan et al., 2011). One hour before the experiments, 1ml fresh medium (37°C) was changed in the 96-well plates (Coster, Corning, NY). In this experiment the cells were cultured for 24h.

3.9.2 Proliferation assay

For detecting the effect of plant extracts on RAW264.7 cell proliferation, the assay was performed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (MTT) assay (Merck, Germany). The MTT test measures the ability of the cells to transform MTT to formazan. The cells were plated in 96-well tissue culture plates at a density of 5×10^5 cells / ml, 5000cell/ well in complete DMEM medium and incubated in triplicate in a 96-well plate in a final volume of 100µl for 24h at 37°C and 5% CO₂ (Gauley & Pisetsky, 2010). Cells were stimulated with plant crude extract and all fractions were at a final concentration (25, 50, 100, 200, 400, 800, and 1000) µg/ml and incubated for 24h at 37° C and 5% CO₂, then 10µl from 5mg/ml in phosphate buffered saline MTT solution was added to each well. After 4h of incubation at 37° C the spent media and MTT were aspirated before the addition of 100 µl of dimethyl sulfoxide (DMSO) (Fisher Scientific, UK) to dissolve the yellow MTT tetrazolium salt produced metabolically and change to purple MTT formazan salt.

The amount of MTT formazan salt produced is proportional to the amount of viable cells. The cell proliferation rate is determined by measuring the absorbance at a wavelength of 570 nm with the microplate reader (Groesdonk et al., 2006; Guan et al., 2011).

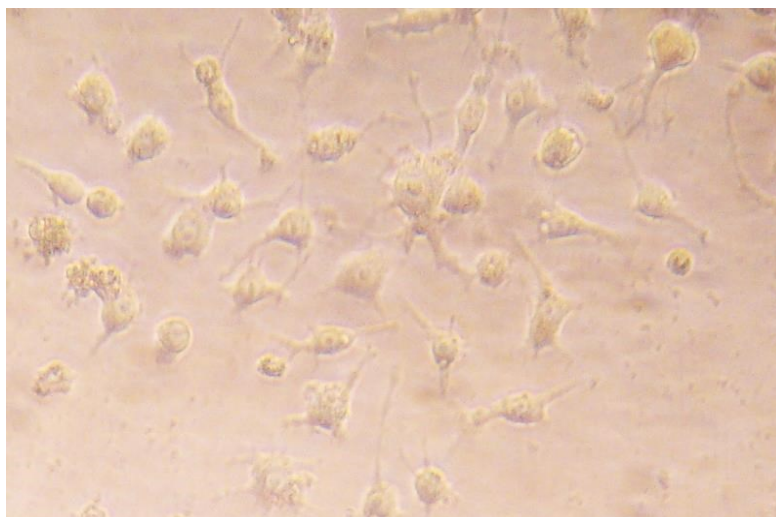


Figure 3.2: A murine macrophage cell line RAW264.7

3.9.3 *In vitro* stimulation for intracellular cytokine production

To evaluate the ability of plant extracts in the stimulation of the RAW 264.7 cell to produce cytokine, RAW264.7 cells (1×10^6 cells /ml, 6 ml /well) were incubated in complete DMEM medium for 24 h at 37°C in 5% CO₂ (Gambelli et al., 2004). Then the cells were stimulated with LPS (1 µg/ml) (*Escherichia coli* 055: B5, Difco, Detroit, MI, USA), (1 µl/ml) brefeldin A (BD GolgiPlug™) and (100 µg/ml) of two plant crude extracts, *P. macrocarpa* fraction1 100 µg/ml (P.m F1) and then *T. crispa* fraction2. One hundred µg/ml (*T. c* F2) were incubated for 6 h (Carahera et al., 2000; Guan et al., 2011). Before activation with LPS (1 µg/ml), the medium was removed and the cells were washed with 5 ml of PBS and replenished with a complete medium (Connelly et al., 2001). Cells stimulated with LPS (1 µg/ml) alone were used as control (Gambelli et al., 2004) and after the incubation period, cells were washed twice with PBS and re-suspended in 0.5 ml of staining buffer PBS containing (1% FBS and 0.09% (w/v) sodium azide) (Carahera et al., 2000).

3.9.4 Flow cytometry immunostaining of intracellular cytokines

Cells were re-suspended with a density of 10^7 cells/ ml, 100 μ l of the suspension cells were aliquoted into tubes for staining. To reduce nonspecific immunofluorescent staining, suspension cells were pre-incubated with mouse BD Fc BlockTM purified anti mouse CD16/CD32 mAb 2.4G2 (BD Fc BlockTM; Cat.No.553141) (1 μ g/ 10^6 cells in 100 μ l) at 4°C for 15 minutes. The cells were then fixed / permeabilized with 250 μ l for 20min at 4°C of fixation/ permeabilization solution (BD Cytofix/Cytoperm plus Fixation/Permeabilization, BD Golgi PlugTM protein transport inhibitor, Cat.No.555028). Cells were washed two times in 1ml of 1 \times BD Perm/WashTM buffer, 250 \times g and 5min at 4°C. Fixed/permeabilized cells were re-suspended in 50 μ l of BD Perm/WashTM buffer and incubated at 4°C for 30 minutes in the dark with a fluorochrome-conjugated anti-cytokine antibody: PE Anti-Mouse IL-6 (≤ 0.25 μ g/million cells, BD Cat. No: 554401), FITC rat anti-mouse IFN- γ (≤ 0.5 μ g/ /million cells, BD Cat. No: 554411) and APC rat anti-mouse IL-8 (≤ 0.5 μ g/million cells, BD Cat. No: FAB2164A). After incubation, cells were washed two times with 1 \times BD Perm/WashTM buffer (1 ml/wash) and re-suspended in Staining Buffer prior to the flow cytometric analysis.

3.9.5 Flow cytometric analysis

The samples were analyzed with gated on the two-dimensional forward and side scatter of the flow cytometer and fluorescence intensity was analyzed with at least 10,000 cells collected for each sample. The cytokine analysis was carried out with a FACS Canto II flow cytometer and FACSDiva version 6.1.3 software (BD Biosciences).

3.10 Investigation of active constituents

High-resolution mass spectrometry was used for the identification and characterization of active constituents in the fractions that have an effect on the proliferation and stimulation of the RAW264.7 cell, *P. macrocarpa* fraction1 (P.m F1) and *T. crispera* fraction 2 (T.c F2). High-resolution mass spectra were recorded on an LC-Mass (Agilent technologies 6530 Accurate-Mass QTOF LC/MC) using MeOH-Water (40:60) as eluent. With the high resolution mass spectra, the identity of the extracted active compounds could be confirmed due to the $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ and $[M+NH_3]^+$ (Di et al., 2013; Salman et al., 2013).

3.11 Statistical analysis

The value is communicated as mean \pm standard deviation (SD). The statistical examination of data was done through one-way analysis of variance (ANOVA), Univariate analysis of variance and Post Hoc LSD test (comparing the treated groups with control). Significance was determined using a *P* value of $P \leq 0.05$ compared with control.

CHAPTER FOUR

4 RESULT

4.1 Extraction of antioxidant compounds

The *P. macrocarpa* and *T. crispa* were extracted according to the method described in (Sec 3.1). 13.5g viscous dark brown was the yield from 200g powder of *P. macrocarpa* and 34.88g viscous dark green was the yield from 200g powder of *T. crispa*.

4.2 Quantitation and evaluation of antioxidant contents and activity

The antioxidant activity of *P. macrocarpa* and *T. crispa* was evaluated by FRAP and DPPH test as shown in Table (4.1). The quantity of total phenolic and total flavonoid contents were presented. In Table (4.1), the *P. macrocarpa* extract has a statically significant $P \leq 0.05$ high antioxidant potential when compared with *T. crispa* and standard ascorbic acid. The FRAP value of *P. macrocarpa* extract is $(17618.52 \pm 2115.9) \mu\text{mol Fe}^{+2}/\text{mg}$, while for *T. crispa* and standard ascorbic acid are $(11011.11 \pm 1145.42, 7951.85 \pm 330.42) \mu\text{mol Fe}^{+2}/\text{mg}$ respectively. In addition, the total antioxidant activity was assessed using a DPPH test that detected DPPH inhibition at % (86.79 ± 0.4) , as well as IC_{50} (14 $\mu\text{g}/\text{ml}$) for *P. macrocarpa*, 55.79 ± 7.995 ; IC_{50} (22 $\mu\text{g}/\text{ml}$) for *T. crispa* and 69.03 ± 9.271 ; IC_{50} (19 $\mu\text{g}/\text{ml}$) for ascorbic acid. Results reveal the total phenolic content of *P. macrocarpa* extract is $(548.51 \pm 76.51 \text{ mg GAE}/\text{g})$ of dry fruit weight and the total flavonoids content was $(213.16 \pm 1.31 \text{ mg QE}/\text{g})$ of dry fruit weight, while total phenolic content is $(84.598 \pm 7.82 \text{ mg GAE}/\text{g})$ and total flavonoid content $(62.07 \pm 39.76 \text{ mg QE}/\text{g})$ in the *T. crispa* stem extract as shown in Table 4.1.

Table 4.1: Antioxidant activity values, total phenolic and total flavonoid contents for *P. macrocarpa* and *T. crispa*.

	FRAP assay μmol Fe+2/mg	DPPH inhibition%	IC50 μg/ ml	Total phenolic contents mg GAE/g	Total flavonoid contents mgTE/g
Ascorbic acid	7951.85 ± 330.42	69.03 ± 9.3	19		
<i>P. macrocarpa</i>	17618.52 ± 2115.9*	86.79±0.4*	14	548.51±76.51	84.598 ± 7.82
<i>T. crispa</i>	11011.11 ± 1145.42	55.79 ± 7.9	22	213.16±1.31	62.07 ± 39.76

Values are expressed as mean ± standard, FRAP value is expressed as Fe⁺² equivalents. IC50 value for the DPPH assay is expressed as μg/ml. Total phenolic content is expressed as Gallic acid equivalent (GAE). Total flavonoid content is expressed as Trolox equivalents (TE). *Significant ($P \leq 0.05$) compared with ascorbic acid.

4.3 Acute toxicity study

During the 14-day experimental period, no records of death or changes to skin and hair in both sexes of rats among the groups administered with doses of both plant extracts (50, 500, and 2000 mg/kg) were observed. In the groups that were treated with *P. macrocarpa*, histological examination and biochemical tests of the kidney and liver conducted at the end of the study did not display any symptom of poisonous substance (Figure 4.1). In groups treated with *T. crispa*, biochemical tests of the kidney and liver showed normal levels, except for alkaline phosphatase which was significantly elevated ($P \leq 0.05$) at doses of 500 and 2000 mg/kg in the female and at all dose groups in the male rats compared with the vehicle control group (Tables 4.2, 4.3, 4.4 and 4.5). In the histological examination (Figure 4.2), the results show that the rats administered with 500 and 2000 mg/kg doses exhibited normal hepatic structure but minimal inflammatory cell infiltration was associated with mild congestion in the portal area. The few blood vessels that were congested, indicated the resolution stage from hepatitis. Based on this strict examination, the LD50 value of *T. crispa* was more than 2000 mg/kg body weight.

Table 4.2: Effect of *P. macrocarpa* & *T. crispa* on the value of liver biochemical parameters in the female rat

Groups	Total protein g/l	Albumin g/l	Globulin g/l	alkaline phosphatase IU/l	aspartate aminotransferase IU/l	alanine aminotransferase IU/l
Vehicle control	69.7 ± 2.07	13.3 ± 1.03	56.3 ± 2.7	64.5 ± 7.89	174.3 ± 28.7	38 ± 5.9
<i>P. macrocarpa</i> 50mg/kg	69.17 ± 5.2	12.17 ± 1.9	57.7 ± 3.27	71.8 ± 14.27	179.2 ± 19.4	44.7 ± 6.98
<i>P. macrocarpa</i> 500mg/kg	68.8 ± 4.5	14.5 ± 1.3	54.5 ± 3.2	52.7 ± 10.6	168.7 ± 32.3	42.2 ± 6.9
<i>P. macrocarpa</i> 2000mg/kg	69.8 ± 4.3	13.7 ± 2.25	56.17 ± 2.8	70.7 ± 16.7	177.7 ± 42.02	38.7 ± 4.8
<i>T. crispa</i> 50mg/kg	69.2 ± 3.9	13 ± 0.9	56.5 ± 3.2	73.5 ± 19.17	179.8 ± 20.6	38.8 ± 4.02
<i>T. crispa</i> 500mg/kg	69.3 ± 1.4	14.7 ± 0.5	54.7 ± 1.4	96 ± 14.5*	198.5 ± 21.8	39.7 ± 6.08
<i>T. crispa</i> 2000mg/kg	69.7 ± 3.8	14.7 ± 1.9	55 ± 2.2	92.8 ± 19.1*	181 ± 21.8	39.5 ± 6.05

*Significant $P \leq 0.05$, Results are presented as mean ± SD. 6rats/group.

Table 4.3: Effect of *P. macrocarpa* & *T. crista* on the value of liver biochemical parameters in the male rat

Groups	Total protein g/l	Albumin g/l	Globulin g/l	Alkaline phosphatase IU/l	Aspartate aminotransferase IU/l	Alanine aminotransferase IU/l
Vehicle control	60 ± 1.8	12 ± 0.89	48.5 ± 1.4	145.92 ± 29.9	171.8 ± 32.95	52.5 ± 1.34
<i>P. macrocarpa</i> 50mg/kg	61.8 ± 4.02	12.17 ± 0.76	51.3 ± 4.13	197.17 ± 57.1	183.8 ± 14.3	45.8 ± 6.9
<i>P. macrocarpa</i> 500mg/kg	63.5 ± 4.04	13 ± 1.09	51.3 ± 4.6	182 ± 34.2	193 ± 23.14	50 ± 7.7
<i>P. macrocarpa</i> 2000mg/kg	61.17 ± 0.41	13 ± 0.9	48.17 ± 1.17	171 ± 44.8	177.7 ± 22.8	54.8 ± 13.03
<i>T. crista</i> 50mg/kg	63 ± 4.97	12.7 ± 1.2	50.7 ± 3.8	215.2 ± 70.08*	177.3 ± 15.1	47.08 ± 8.6
<i>T. crista</i> 500mg/kg	63.5 ± 5.3	13.2 ± 1.3	50.7 ± 4.3	257.7 ± 14.4*	197.8 ± 33.6	56 ± 1.5
<i>T. crista</i> 2000mg/kg	63 ± 4.9	13.2 ± 1.3	50.2 ± 4.1	261.8 ± 12.96*	193.08 ± 31.7	56.5 ± 1.8

*Significant $P \leq 0.05$, Results are presented as mean ± SD. 6rats/group.

Table 4.4: Effect of *P. macrocarpa* & *T. crispa* on the value of kidney biochemical parameters in the female rat

Groups	Sodium mmol/L	Potassium mmol/L	Chloride mmol/L	Carbone Dioxide mmol/L	Urea mmol/L	Creatinine umol/L
Vehicle control	139.7 ± 1.2	4.5 ± 0.2	98.5 ± 10.7	21.6 ± 1.79	5.9 ± 0.6	41.7 ± 14.8
<i>P. macrocarpa</i> 50mg/kg	140.3 ± 1.3	4.8 ± 0.08	102.17 ± 12.5	18.2 ± 7.3	5.7 ± 1.08	39.5 ± 7.6
<i>P. macrocarpa</i> 500mg/kg	137.8 ± 3.5	4.6 ± 0.3	77.17 ± 34.6	24.1 ± 2.5	8.52 ± 4.8	40.5 ± 13.35
<i>P. macrocarpa</i> 2000mg/kg	139.8 ± 1.17	4.7 ± 0.4	100.3 ± 11.6	23.4 ± 1.07	6.8 ± 0.5	32.7 ± 7.4
<i>T. crispa</i> 50mg/kg	139.7 ± 1.9	4.7 ± 0.2	99.7 ± 11.3	20.1 ± 2.6	5.4 ± 0.9	40.8 ± 9.2
<i>T. crispa</i> 500mg/kg	140.3 ± 1.2	4.6 ± 0.2	99.2 ± 10.9	22.3 ± 2.9	6.2 ± 1.4	35 ± 8.4
<i>T. crispa</i> 2000mg/kg	140 ± 1.5	4.5 ± 0.3	99.5 ± 11.1	22.8 ± 2.1	6.6 ± 0.6	40.3 ± 6.5

Results are presented as mean ± SD. 6rats/group.

Table 4.5: Effect of *P. macrocarpa* & *T. crispa* on the value of kidney biochemical parameters in the male rat

Groups	Sodium mmol/L	Potassium mmol/L	Chloride mmol/L	Carbone Dioxide mmol/L	Urea mmol/L	Creatinine umol/L
Vehicle control	140.8 ± 2.14	5.05 ± 0.05	102.3 ± 0.5	22.2 ± 1.07	3.95 ± 0.2	31.5 ± 4.03
<i>P. macrocarpa</i> 50mg/kg	141 ± 0.6	4.7 ± 0.33	103.9 ± 1.61	23.65 ± 1.6	4.6 ± 0.84	29.8 ± 6.5
<i>P. macrocarpa</i> 500mg/kg	140.17 ± 0.98	4.8 ± 0.3	103.8 ± 2.04	24.1 ± 1.44	4.2 ± 0.3	24.42 ± 6.8
<i>P. macrocarpa</i> 2000mg/kg	142.17 ± 1.4	5.13 ± 0.3	104.3 ± 1.8	24.7 ± 2.91	4.5 ± 0.7	29 ± 3.8
<i>T. crispa</i> 50mg/kg	140.5 ± 1.6	4.9 ± 0.4	98 ± 10.4	22.7 ± 1.7	4.7 ± 1.1	34.8 ± 10.6
<i>T. crispa</i> 500mg/kg	140.8 ± 1.5	4.7 ± 0.3	98 ± 10.4	22.7 ± 1.7	4.7 ± 1.07	33.3 ± 11.3
<i>T. crispa</i> 2000mg/kg	142.5 ± 1.9	4.9 ± 0.4	99 ± 10.9	23.4 ± 2.5	4.7 ± 1.1	35.3 ± 8.3

Results are presented as mean ± SD. 6rats/group.

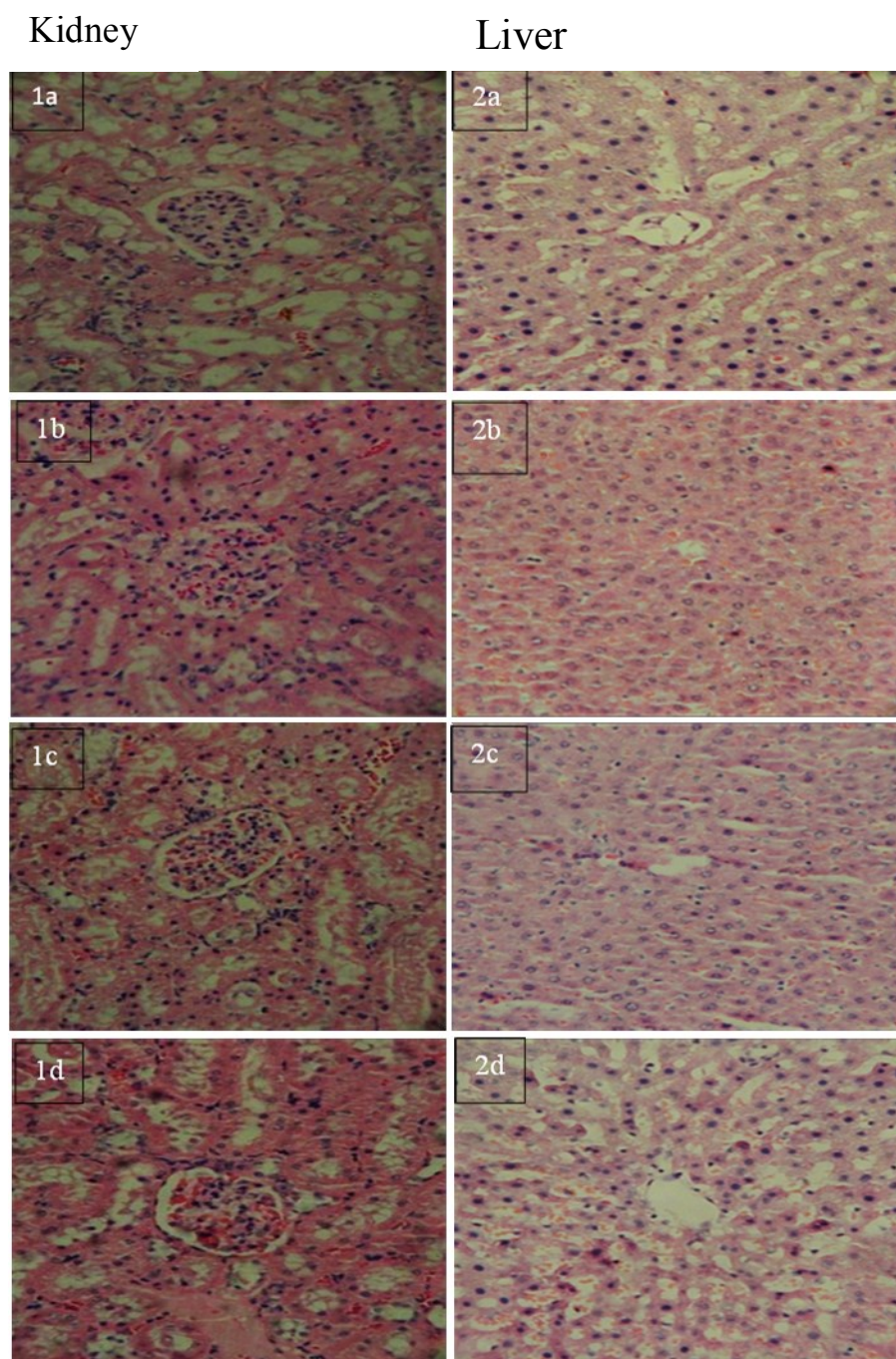


Figure 4.1: Effect of *P. macrocarpa* on liver and kidney. Rat (1a and 2a) treated with 5ml/kg vehicle as control group. Rat (1b and 2b) treated with 50mg/kg. Rat (1c and 2c) treated with 500mg/kg and rat (1d and 2d) treated with 2000mg/kg) of *P. macrocarpa* (hematoxylin and eosin staining, (10x).

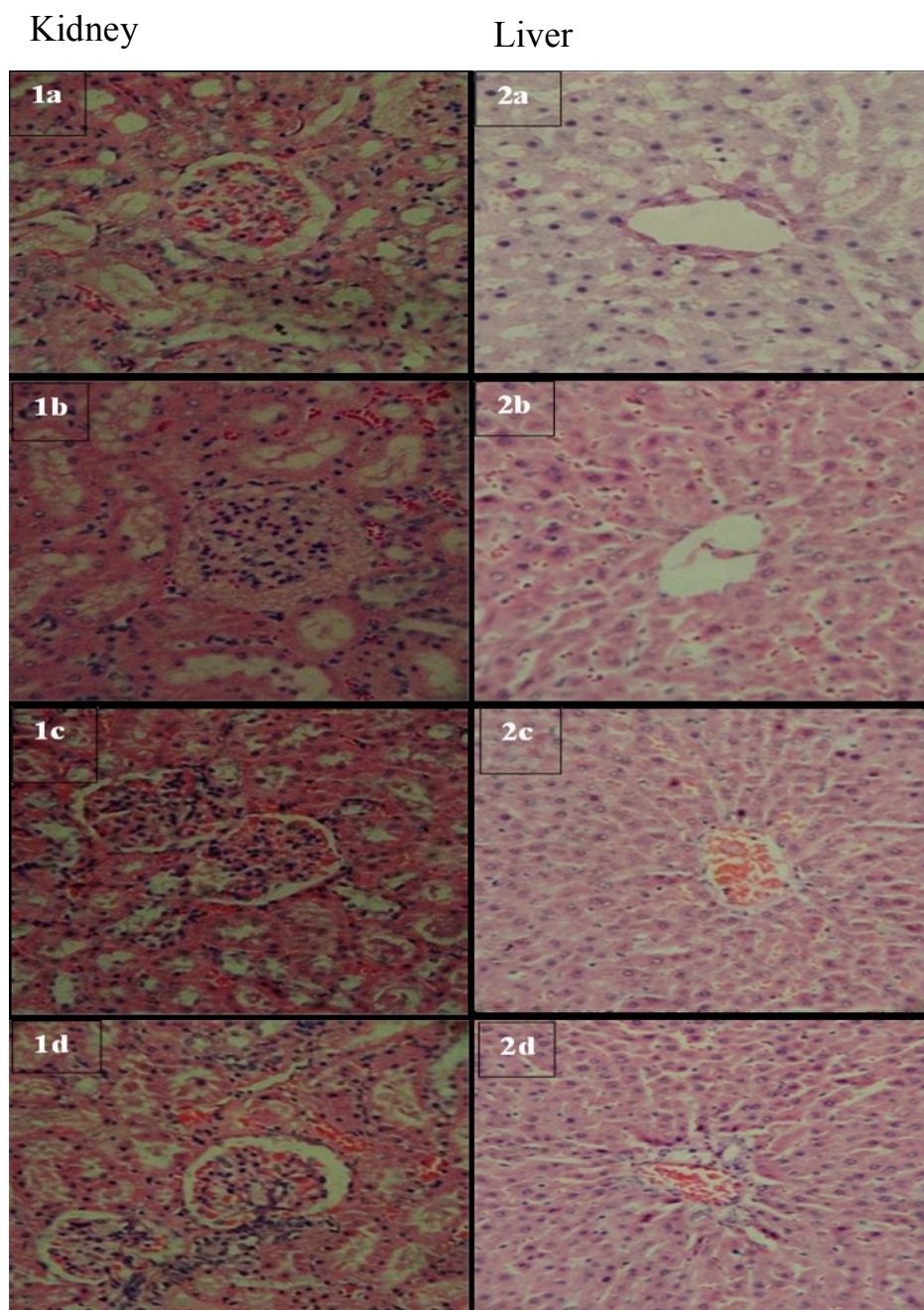


Figure 4.2: Effect of *T.crispa* on liver and kidney. Rat (1a and 2a) treated with 5ml/kg vehicle as control group. Rat (1b and 2b) treated with 50mg/kg. Rat (1c and 2c) treated with 500mg/kg and rat (1d and 2d) treated with 2000mg/kg) of *T.crispa* (hematoxylin and eosin staining, (10x).

4.4 Gastroprotective ability of the plant extract

The gastroprotective effect and the enhancement of the mechanism defense of gastric ulcer by *P. macrocarpa* or *T. crispa* were investigated through the induction of gastric ulcer in rats using ethanol as a necrotizing agent. This scheme resulted in mucosal injury characterized by submucosal edema, increased secretory products of the cell, and disturbances in microcirculation.

Pretreatment with ethanol extract of *P. macrocarpa* or of *T. crispa* stems at doses of 100, 200, 500 and 1000 mg/kg or omeprazole at 20 mg/kg inhibited the formation of gastric ulcer lesions in the rats. The intensity of the ulcer was detected by a statistically significant ($P \leq 0.05$) ulcer area in the pretreated groups (*P. macrocarpa*, *T. crispa* or omeprazole), which was lower than that of the ulcer control group. The ulcer inhibition percentage reflected the results of pretreatment with all doses of the *P. macrocarpa* or *T. crispa* and was higher than the ulcer control group (Figure 4.3).

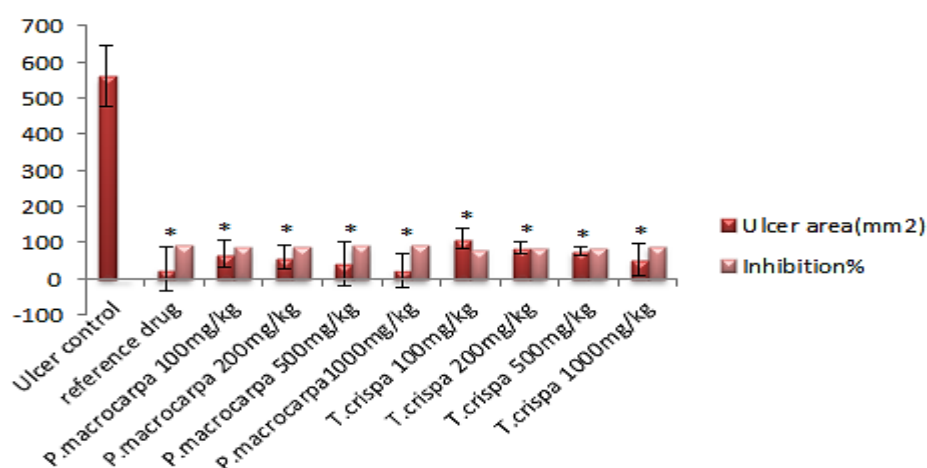


Figure 4.3: Effect of *P. macrocarpa* and *T. crispa* on the ulcer area (values expressed as mean \pm SD) and inhibition %. 6 rats / groups. * Significant ($P \leq 0.05$) when compared with ulcer control group.

The stomach pH in the experimental groups pretreated with *P. macrocarpa*, *T. crispa* or omeprazole increased significantly compared to the ulcer control group $P \leq 0.05$ (Figure 4.4).

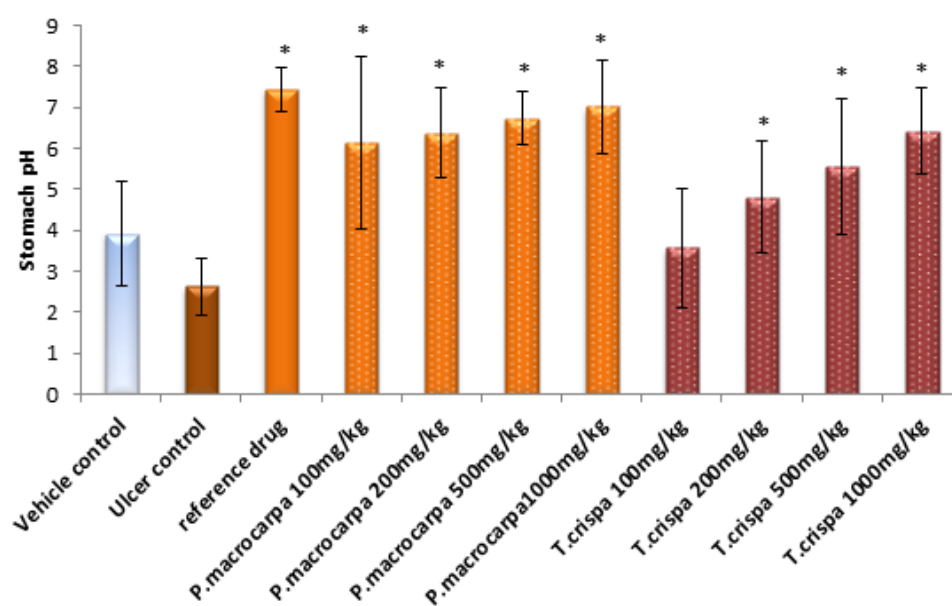


Figure 4.4: Effect of *P. macrocarpa* and *T. crispa* on the stomach pH. Values are presented as mean \pm SD, 6 rats / groups. * Significant ($P \leq 0.05$) when compared with ulcer control group.

4.4.1 Histological estimation of gastric ulcer

The absence of injuries in the gastric mucosa was observed in the vehicle control group. In the gastric mucosa during ulcer control, severe injuries were observed. Ethanol formed widespread observable hemorrhagic necrosis of the gastric mucosa. In addition, damage in the gastric mucosa in rats pretreated with omeprazole (20 mg/kg) were minor compared to injuries in the ulcer control rats. In the pretreatment with *P. macrocarpa* extract at doses of 100 and 200 mg/kg, injuries were observed in the gastric mucosa. However, in rats pretreated with *P. macrocarpa* extracts (500 and 1000 mg/kg) only mild injuries were seen. The extract reduced gastric injuries that were induced by ethanol (Figure 4.5a).

Microscopy analysis results on the severity of the ulcer lesions are presented in (Figure 4.5b). The results show damage throughout the gastric mucosa layer and edema and leukocyte infiltration in the submucosa layer in the ulcer control group. In the histology of the stomach of rats pretreated with *P. macrocarpa* extracts (100 and 200 mg/kg) there was seen a disturbance of the surface epithelium mucosa. Edema and leukocyte penetration of the submucosal stratum was also observed. In the rat pretreated with *P. macrocarpa* extracts (500 and 1000 mg/kg) there was observed a minor disturbance to the epithelium and absence of edema or leukocyte infiltration of the submucosal stratum. The treated samples exhibited better protection from injury caused by ethanol as clearly indicated by the reduced ulcer area, less damage to the gastric mucosa, reduced edema in the submucosa layer and decreased leukocyte infiltration. Therefore, *P. macrocarpa* exhibits gastroprotective influences in a dose dependent way.

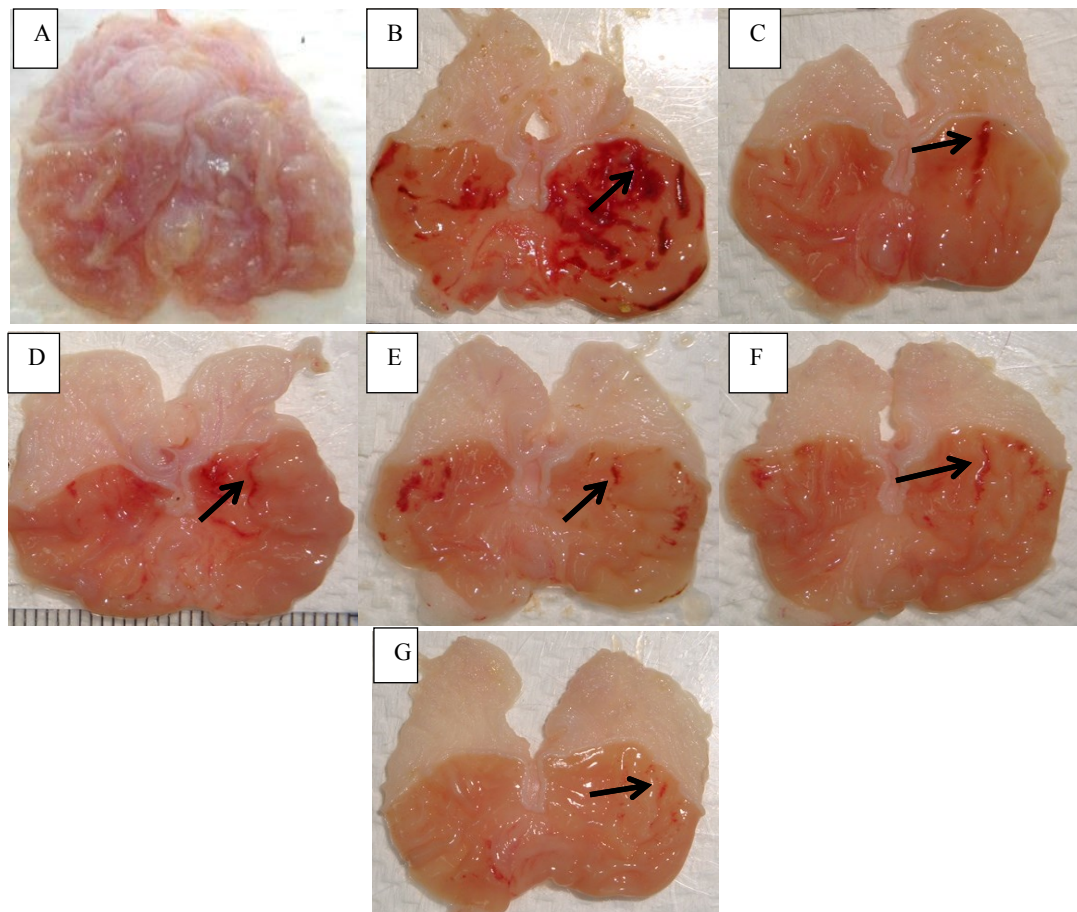


Figure 4.5a: Effect of *P. macrocarpa* on the gross appearance of the gastric mucosa in rats. (A) Rats pretreated with 5ml/kg Teen20, no injuries to the gastric mucosa are seen. (B) Rats pretreated with 5ml/kg Teen20 + ethanol; severe injuries to the gastric mucosa are seen. Ethanol produced extensive visible hemorrhagic necrosis of gastric mucosa. (C) Rats pretreated with omeprazole (20mg/kg); mild injuries are seen in the mucosa layer. (D & E) rats pretreated with *P. macrocarpa* extract (100 & 200 mg/kg) consecutively; injuries are seen in the gastric mucosa. (F&G) rats pretreated with *P. macrocarpa* extract (500 & 1000 mg/kg) consecutively; mild injuries are seen in the gastric mucosa. The black arrow indicates mucosa injury.

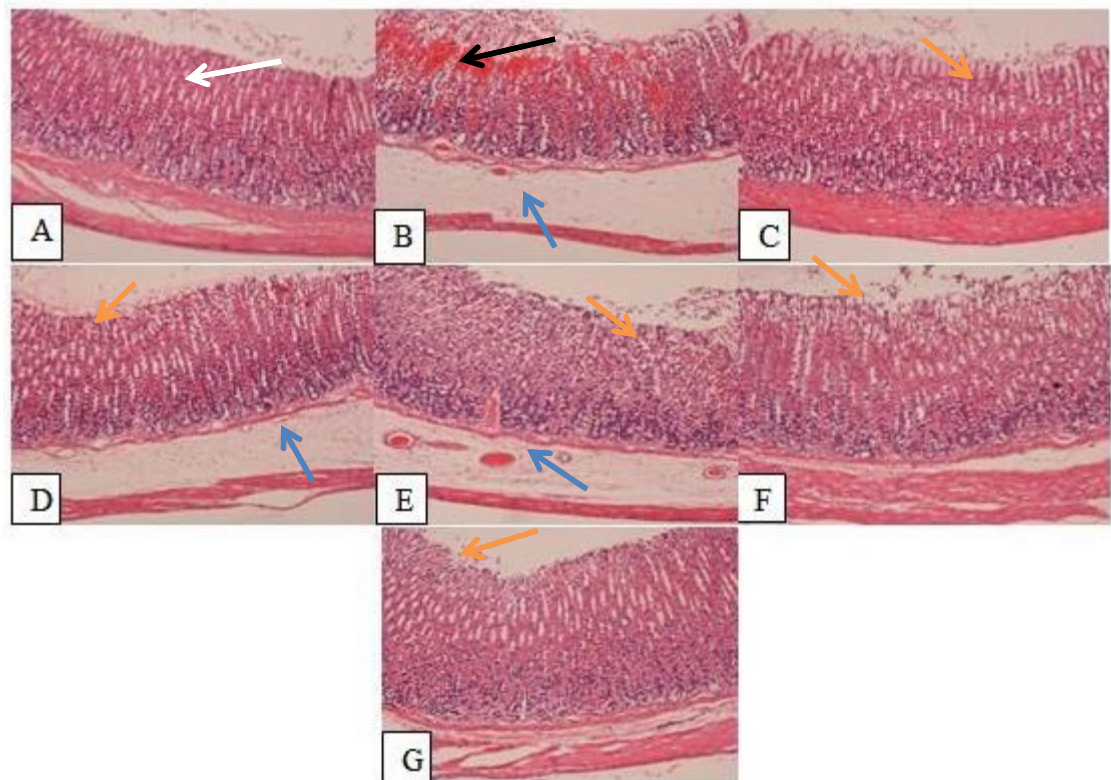


Figure 4.5b: Effect of *P. macrocarpa* on the histological appearance of ethanol-induced gastric mucosal damage in rat. (A) Rats pretreated with 5mL/kg of Tween20. No injuries to the gastric mucosa are seen (white arrow). (B) Rats pretreated with 5mL/kg of Tween20 + 95% ethanol. There is severe disruption to the surface epithelium and necrotic lesions (black arrow) penetrating deeply into the mucosa and extensive edema of the submucosal layer and leucocyte infiltration are present (blue arrow). (C) Rats pretreated with omeprazole (20mg/kg). Mild disruption of the surface epithelium mucosa is seen (orang arrow). (D), (E) rat pretreated with *P. macrocarpa* extract (100, 200mg/kg) consecutively; mild disruption of the surface epithelium mucosa is seen. There is edema and leucocyte infiltration of the submucosal layer. (F) Rat pretreated with *P. macrocarpa* extract (500mg/kg); mild disruption to the surface epithelium. (G) Rat pretreated with *P. macrocarpa* extract (1000mg/kg); minor disruption to the surface epithelium and no edema or leucocyte infiltration of the submucosal layer (H&E stain 10 ×).

A deficiency of injuries in the gastric mucosa was observed in the vehicle control. In the gastric mucosa in ulcer control, severe injuries were observed. Ethanol formed widespread observable hemorrhagic necrosis of the gastric mucosa. In addition, damage in the gastric mucosa in rats pretreated with omeprazole (20 mg/kg) was minor compared to injuries in the ulcer control rats. The group of rats pretreated with *T. crispa* extract at doses of 100 and 200 mg/kg were observed to have injuries in the gastric mucosa. However, rats pretreated with *T. crispa* extracts (500 and 1000 mg/kg) only had slight damage. The extract reduced the development of gastric injuries that were induced by ethanol (Figure 4.6a).

Microscopy analysis results of the severity of ulcer lesions are presented in (Figure 4.6b). Results show damage throughout the gastric mucosa layer and edema and leukocyte infiltration in the submucosa layer of the ulcer control group. In the histology of the stomachs of the rats pretreated with *T. crispa* extracts, (100 and 200 mg/kg) there was observed a disturbance of the surface epithelium mucosa. In addition edema and leukocyte infiltrate of the submucosal layer were observed. In the rats pretreated with *T. crispa* extracts (500 and 1000 mg/kg) there was observed a slight disturbance on the surface of the epithelium and an absence of edema or leukocyte penetration of the submucosal layer. The treated samples exhibited better protection from injury caused by ethanol as clearly indicated by the reduced ulcer area, less damage to the gastric mucosa, reduced edema in the submucosal layer and decreased leukocyte infiltration. Therefore, *T. crispa* exhibits gastroprotective effects in a dose dependent manner.

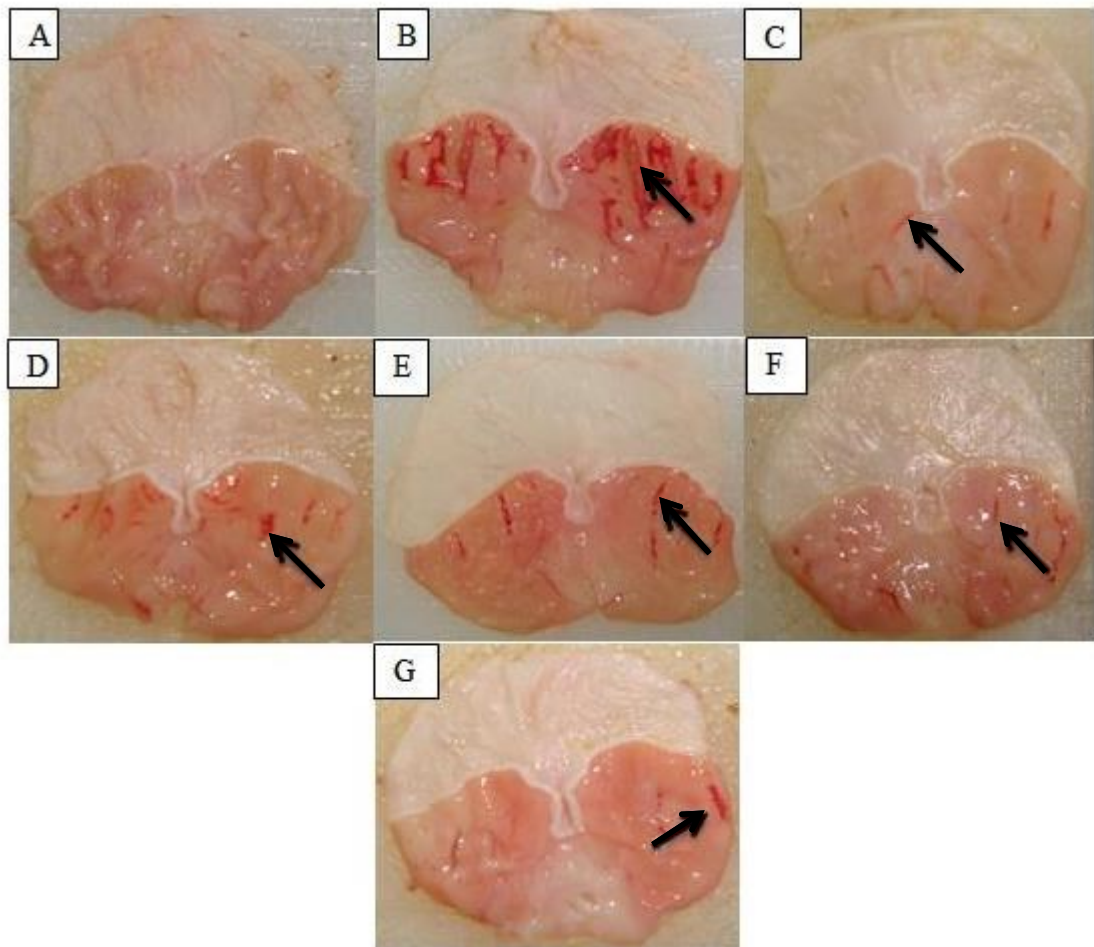


Figure 4.6a: Effect of *T. crispa* on the gross appearance of the gastric mucosa in rats. (A) Rats pretreated with 5ml/kg Tween20, no injuries to the gastric mucosa are seen. (B) Rats pretreated with 5ml/kg Tween20 + 95% ethanol; severe injuries to the gastric mucosa are seen. Ethanol produced extensive visible hemorrhagic necrosis of gastric mucosa. (C) rats pretreated with omeprazole (20mg/kg); mild injuries are seen in the mucosal layer. (D & E) rats pretreated with *T. crispa* extract (100 & 200 mg/kg) consecutively; injuries are seen in the gastric mucosa. (F&G) rats pretreated with *T. crispa* extract (500 & 1000 mg/kg) consecutively; mild injuries are seen in the gastric mucosa. The black arrow indicates mucosal injury.

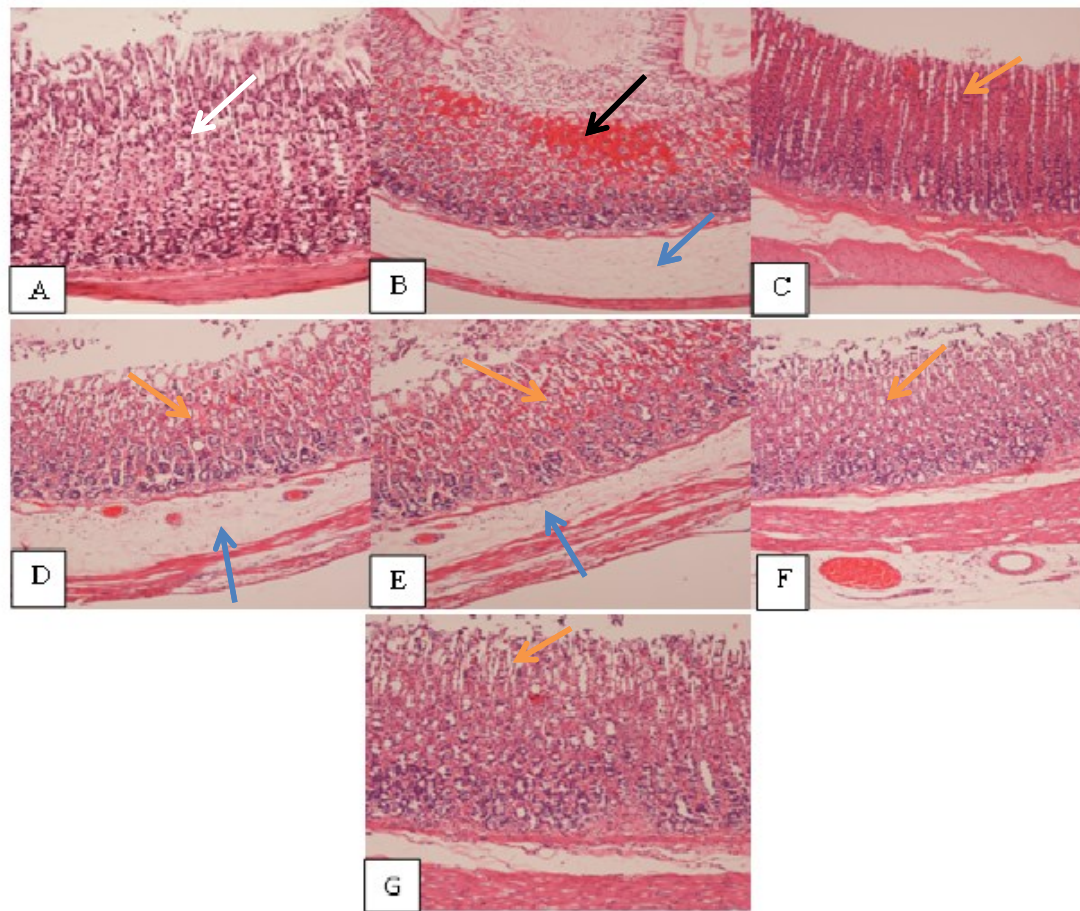


Figure 4.6b: Effect of *T. crispata* on the histological appearance of ethanol-induced gastric mucosal damage in rats. (A) Rat pretreated with 5mL/kg of Tween20. No injuries to the gastric mucosa are seen (white arrow). (B) Rat pretreated with 5mL/kg of Tween20 + 95% ethanol. There is severe disruption to the surface epithelium and necrotic lesions penetrating deeply into the mucosa (black arrow) and extensive edema of submucosal layer and leucocyte infiltration are present (blue arrow). (C) Rat pretreated with omeprazole (20mg/kg). Mild disruption of the surface epithelium mucosa is seen (orange arrow). (D), (E) Rat pretreated with *T. crispata* extract (100, 200mg/kg) consecutively; disruption of the surface epithelium mucosa is seen. There is edema and leucocytes infiltration of the submucosal layer. (F) Rat pretreated with *T. crispata* extract (500mg/kg); mild disruption to the surface epithelium and edema, no leucocyte infiltration of the submucosal layer. (G) Rat pretreated with *T. crispata* extract (1000mg/kg); slight disruption to the surface epithelium and no edema or leucocytes infiltration of the submucosal layer (H&E stain 10 ×).

4.4.2 Evaluation of gastrin, pepsin and PGE2

In groups pretreated with *P. macrocarpa*, *T. crispa* and omeprazole, the results show a significant decrease ($P \leq 0.05$) in gastrin (Figure 4.7) and pepsin (Figure 4.8) levels compared to the ulcer control group. In addition, the PGE2 level in the groups pretreated with *P. macrocarpa* at doses of (100, 200, 500, 1000 mg/kg), omeprazole and *T. crispa* at doses of (500 and 1000 mg/kg) significantly ($P \leq 0.05$) increased compared to the ulcer control group (Figure 4.9).

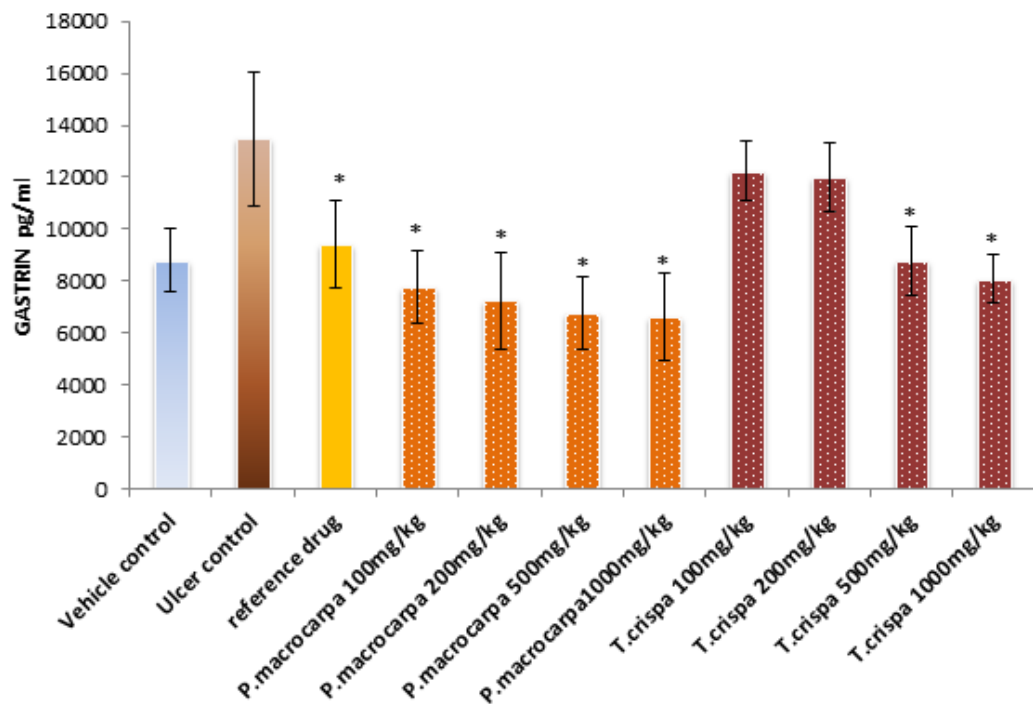


Figure 4.7: Effect of *P. macrocarpa* and *T. crispa* on the level of serum gastrin in ethanol induced gastric ulcer, (6 rats/group). Values are presented as mean \pm SD. * significant at ($P \leq 0.05$) compared with ulcer control group.

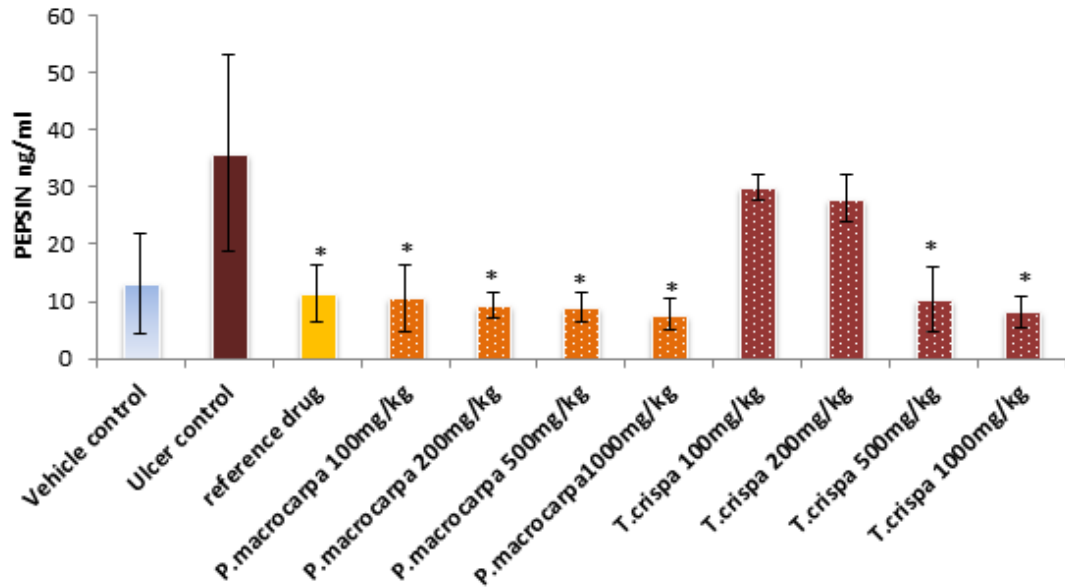


Figure 4.8: Effect of *P. macrocarpa* and *T. crispa* on the level of serum pepsin in ethanol induced gastric ulcer; (6 rats/group). Values are presented as mean \pm SD. * significant at ($P \leq 0.05$) compared with ulcer control group.

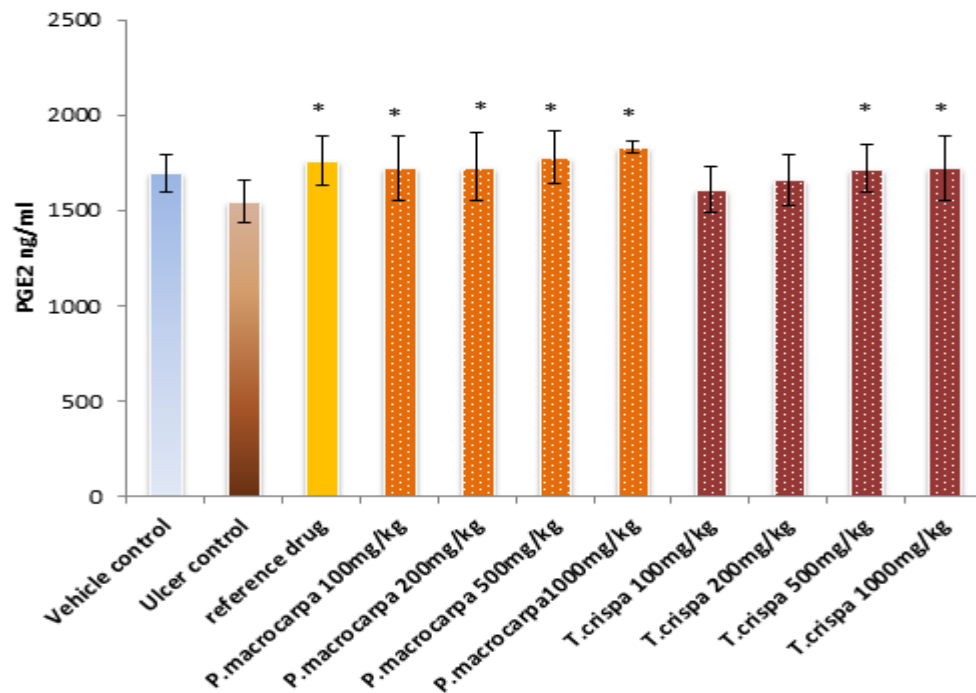


Figure 4.9: Effect of *P. macrocarpa* and *T. crispa* on the level of PGE2 in ethanol induced gastric ulcer, (6 rats/group). Values are presented as mean \pm SD. *significant at ($P \leq 0.05$) compared with ulcer control group.

4.4.3 Estimation of SOD, CAT and MDA level

The effect of *P. macrocarpa* and *T. crispa* on antioxidant enzymes and its gastroprotective role were investigated in the pretreated groups. The results show that the two plants significantly ($P \leq 0.05$) affected SOD (Figure 4.10) and CAT (Figure 4.11) by increasing their levels in all pretreated groups compared to the ulcer control group. Moreover, the results show that the level of MDA significantly decreased ($P \leq 0.05$) in all pretreated groups compared to the ulcer control group (Figure 4.12).

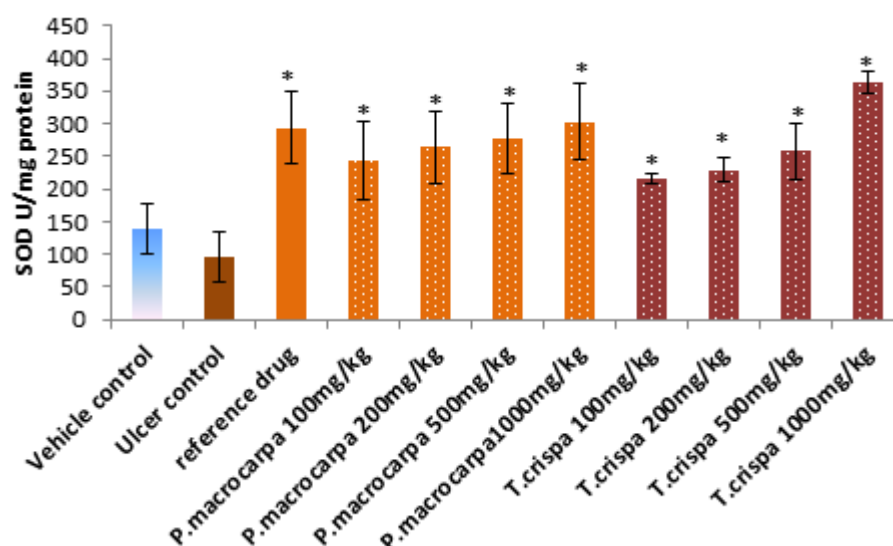


Figure 4.10: Effect of *P. macrocarpa* and *T. crispa* on the level of SOD in ethanol induced gastric ulcer, (6 rats/group). Values are presented as mean \pm SD.*significant at ($P \leq 0.05$) compared with ulcer control group.

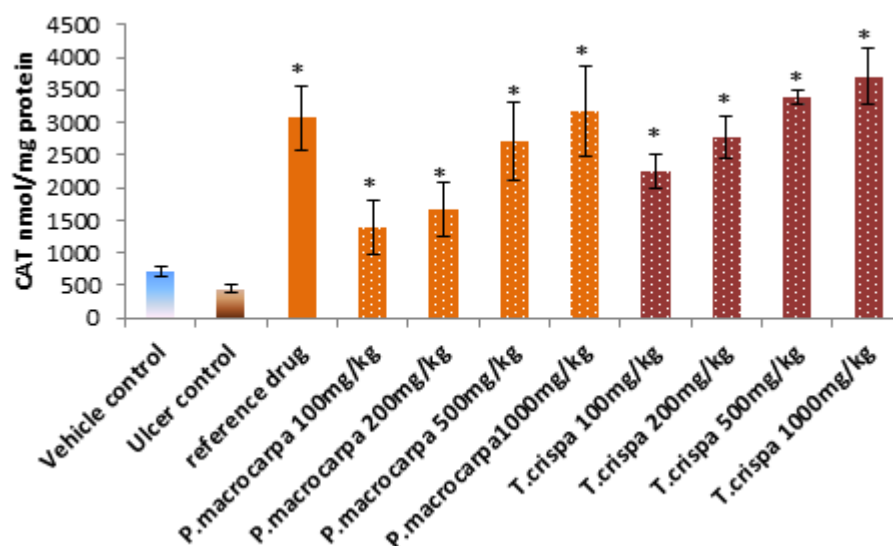


Figure 4.11: Effect of *P. macrocarpa* and *T. crispa* on the level of CAT in ethanol induced gastric ulcer, (6 rats/group). Values are presented as mean \pm SD.*significant ($P \leq 0.05$) compared with ulcer control group.

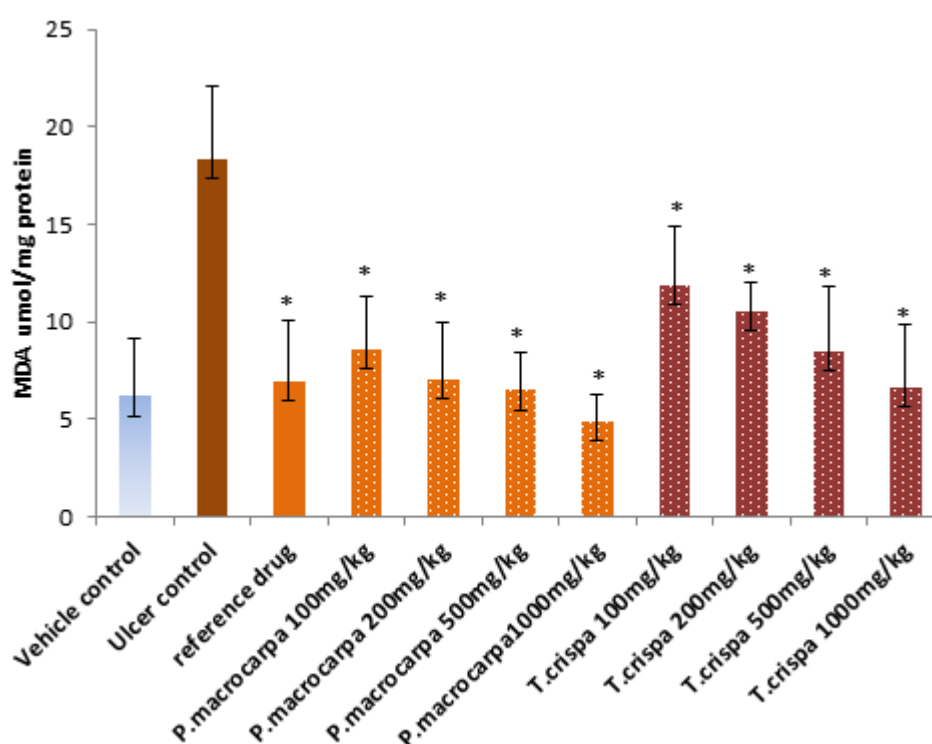


Figure 4.12: Effect of *P. macrocarpa* and *T. crispa* on the level of MDA in ethanol induced gastric ulcer, (6 rats/group). Values are presented as mean \pm SD.*significant ($P \leq 0.05$) compared with ulcer control group.

4.4.4 Estimation of TGF- β 1 and TNF- α

Results show that the level of TGF- β 1 significantly ($P \leq 0.05$) increased in pretreated groups with *P. macrocarpa* and *T. crispa* when compared to ulcer control (Figure 4.13). By contrast, the level of TNF- α significantly decreased ($P \leq 0.05$) compared to the ulcer control group, in groups pretreated with *P. macrocarpa* and *T. crispa* when compared with ulcer control group (Figure 4.14).

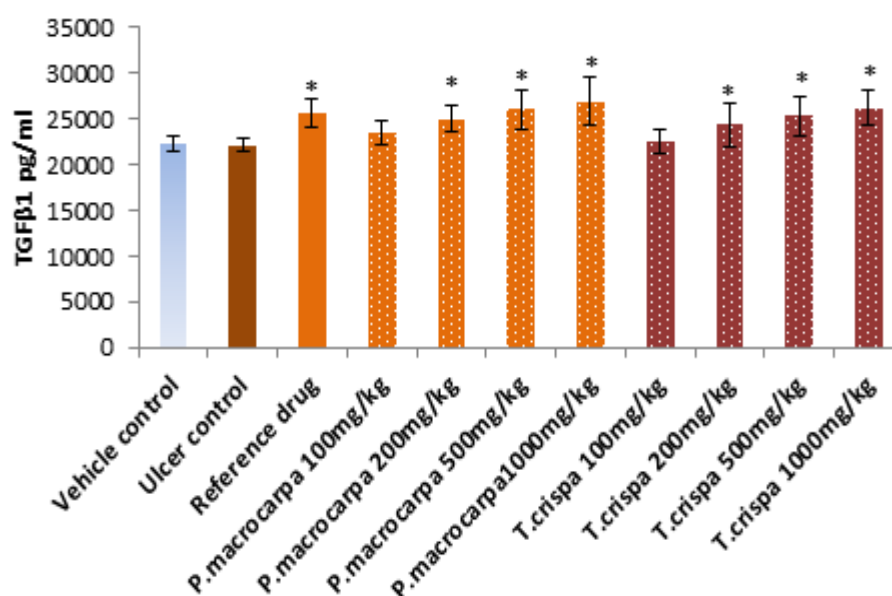


Figure 4.13: Effect of *P. macrocarpa* and *T. crispa* on the level of TGF- β 1 in ethanol induced gastric ulcer, (6 rats/group). Values are presented as mean \pm SD. * significant ($P \leq 0.05$) compared with ulcer control group.

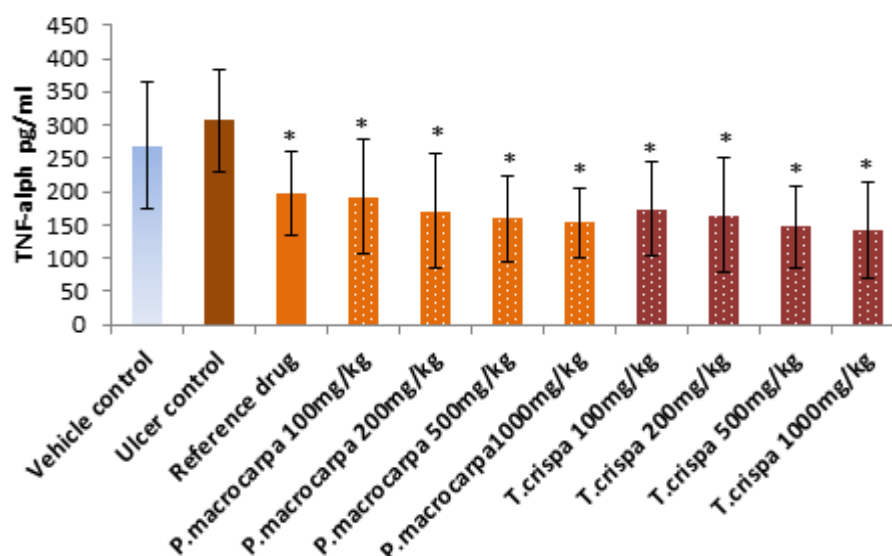


Figure 4.14: Effect of *P. macrocarpa* and *T. crispa* on the level of TNF- α in ethanol induced gastric ulcer, (6 rats/group). Values are presented as mean \pm SD. * significant ($P \leq 0.05$) compared to the ulcer control group.

4.5 Mucus barrier of the protective stomach

The results of the mucus barrier contents presented in Figure 4.15 show statistically ($P \leq 0.05$) higher mucus content in the groups pretreated with *P. macrocarpa* extract, *T. crispa* and omeprazole compared to those in the ulcer control group.

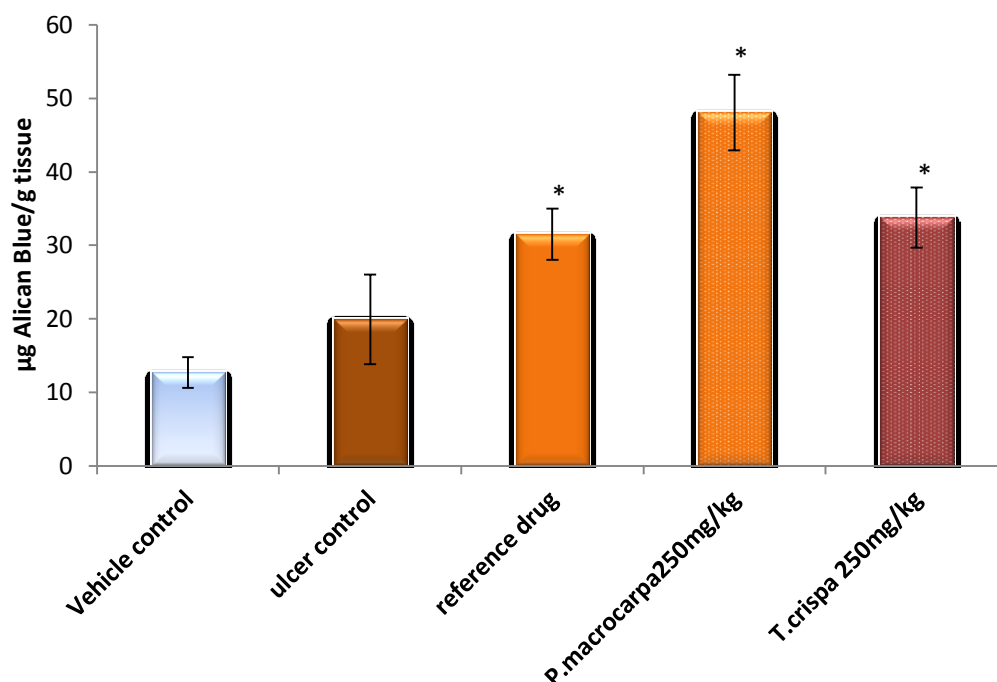


Figure 4.15: Effects of *P. macrocarpa*, *T. crispa* and reference drug (omeprazole) on gastric wall mucus content in ethanol induced ulcer in rats. Values are presented as mean \pm S.D.* significant ($P \leq 0.05$) compared with ulcer control group (6 rats/group).

4.6 Wound healing evaluation parameters

This study was carried out in order to verify the ability of *P. macrocarpa* and *T. crispa* to enhance the wound healing process. The circular excision wound model was employed for assessing the *in vivo* wound healing activity of these medicinal plants. Two percent concentration solution of gum acacia was used to prepare plant extract at doses of 100mg/ml and 200 mg/ml applied to the experimentally created excision wounds of rats.

4.6.1 Wound area contraction

A neutral observer who was unaware of the experimental protocol evaluated the wound healing rate. Macroscopically, the wounds dressed with *P. macrocarpa* or *T. crispa* showed considerable signs of dermal healing and healed significantly faster compared to the vehicle control group (gum acacia in normal saline) on day 15 after the wounds were incurred (Figures 4.16 and 4.17).

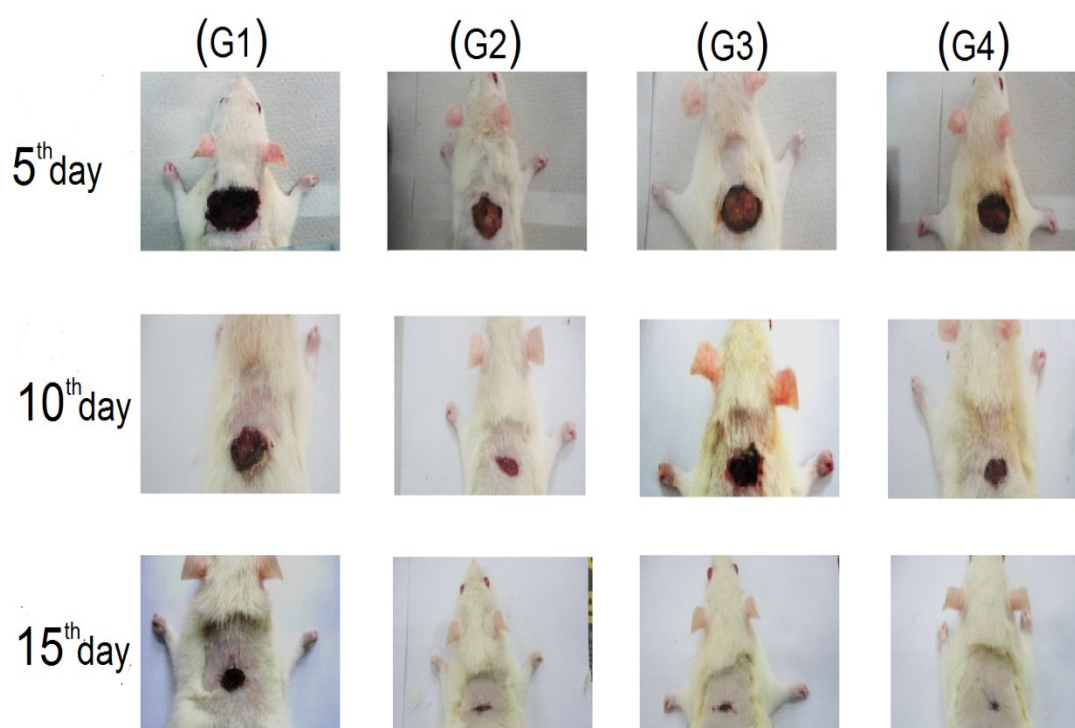


Figure 4.16: Macroscopical appearance of excision wound healing area treatment. (G1) 0.2ml of vehicle control (gum acacia in normal saline, 20mg/ml). (G2) 0.2ml Intracite. (G3) 0.2ml of 100mg/ml of *P. macrocarpa*. (G4) 0.2ml of 200mg/ml of *P. macrocarpa*.

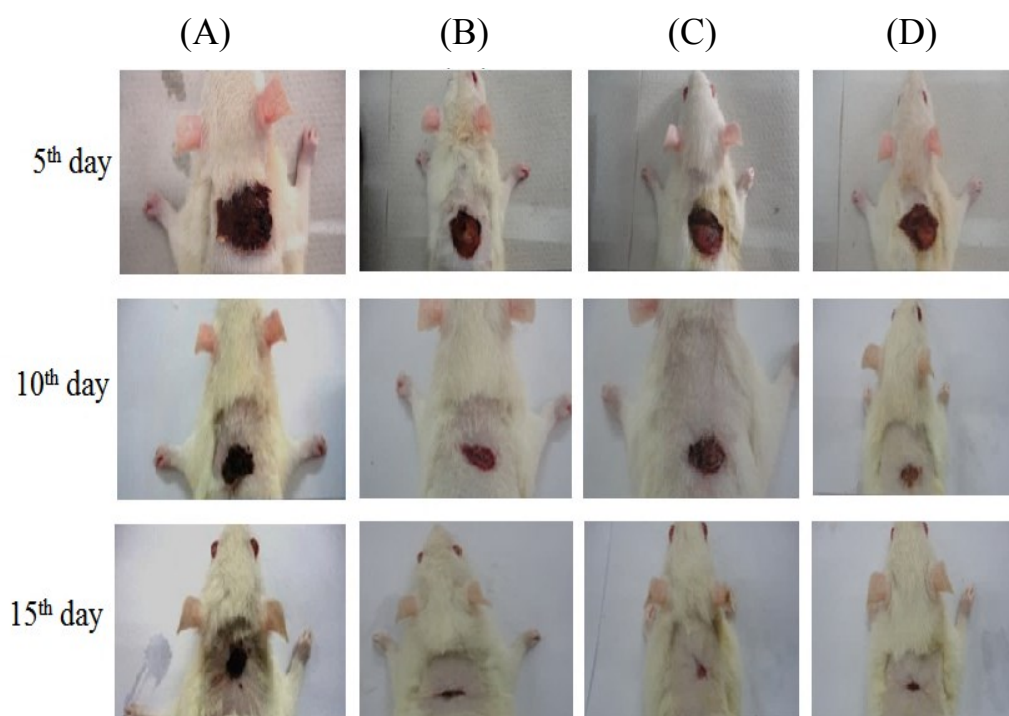


Figure 4.17: Macroscopical appearance of excision wound healing area treatment. (A) 0.2ml of vehicle control (gum acacia in normal saline, 20mg/ml). (B) 0.2ml Intrasisite. (C) 0.2ml of 100mg/ml of *T. crispa*. (D) 0.2ml of 200mg/ml of *T. crispa*.

Measurements of the wound healing progression as induced by the ethanol extract and the reference drug on the vehicle control groups are shown in (Figure 4.18). The wound margin was traced after wound creation using transparent paper and the wound area was measured using graph paper. Wound contraction was measured over an interval of 5 days until the 15th day. In the excision wound model, the wounds of the groups of animals treated with *P. macrocarpa* and *T. crispa* at doses of (100 and 200) mg/ml contracted at a significantly higher rate by Day 15 ($P \leq 0.05$) compared to the vehicle control group.

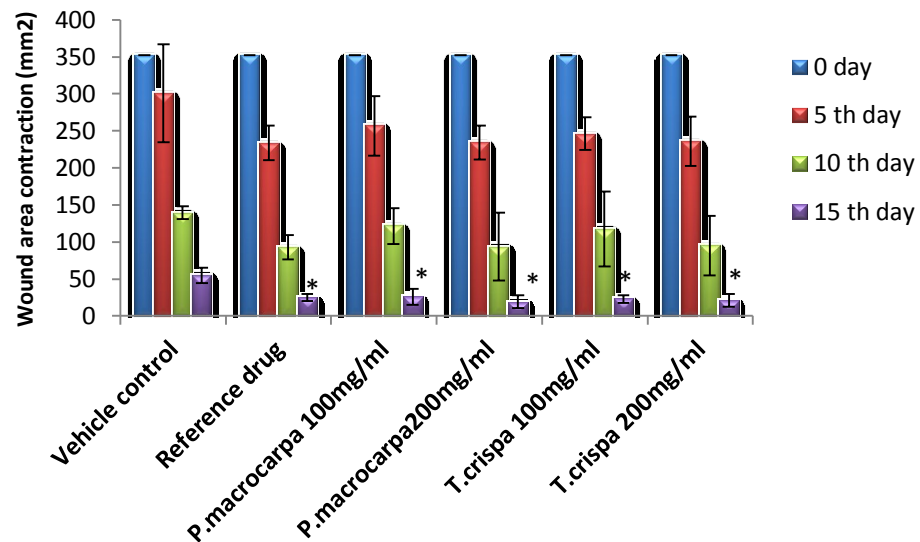


Figure 4.18: Effects of *P.macrocarpa* and *T. crispa* on wound area contraction (mm²) in rats. Values are expressed as mean \pm SD, (5 rats/group). *significant $P \leq 0.05$.

4.6.2 Histopathological evaluation of healed wound area

The histology of the wound tissue on the 15th day after wounding was evaluated by an observer blinded to the experimental protocol. In the *P.macrocarpa* treated groups and *T. crispa* treated groups, the wound enclosure was smaller and the granulation tissues contained comparatively less inflammatory cells and more collagen, fibroblasts and blood proliferating capillaries compared to the vehicle control group (Figures 4.19 and 4.20).

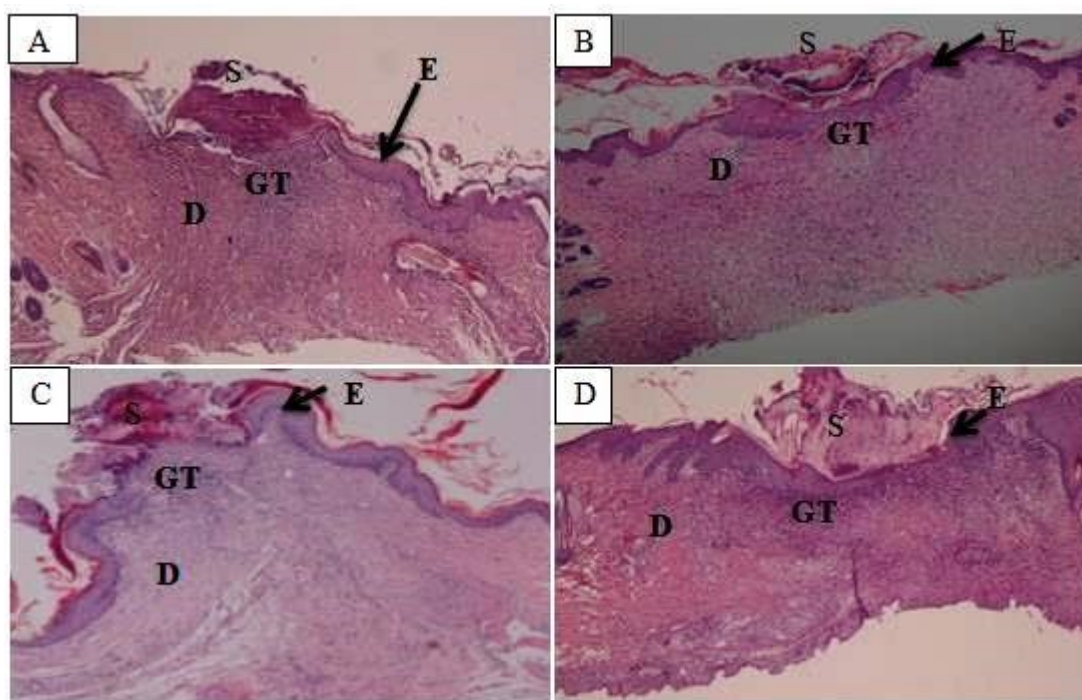


Figure 4.19: hematoxylin and eosin-stained sections of the wound on the 15th day after wounding in rats. (A) 0.2ml of vehicle, gum acacia in normal saline. (B) 0.2ml of Intrasite gel. (C) 0.2ml of *P. macrocarpa* (100mg/ml). (D) 0.2ml of *P. macrocarpa* (200mg/ml). S = Scab, E = Epidermis, GT = granulation tissue. (Magnification 20x)

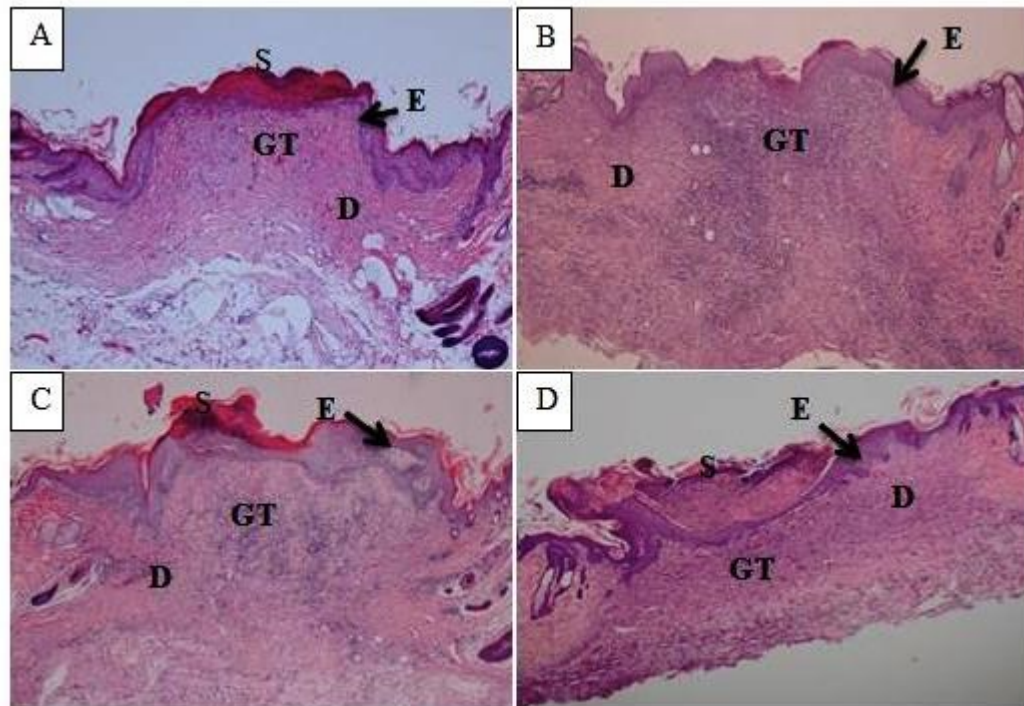


Figure 4.20: hematoxylin and eosin-stained sections of the wound on the 15th day after wounding in rats. (A) 0.2ml of vehicle, gum acacia in normal saline. (B) 0.2ml of Intracel gel. (C) 0.2ml of *T. crispata* (100mg/ml). (D) 0.2ml of *T. crispata* (200mg/ml). S = Scab, E = Epidermis, GT = granulation tissue. (Magnification 20x)

4.6.3 SOD, CAT, and MDA evaluation in the healed wound area

Antioxidant enzymes (SOD and CAT) and MDA (natural product of lipid peroxidation) were estimated in this study to evaluate the progression of the wound healing process and to determine the effect of *P. macrocarpa* and *T. crispata*. Results reveal that the elevated SOD level was significant $P \leq 0.05$ compared to the vehicle control group (Figure 4.21). A similar result was obtained with regards to CAT level ($P \leq 0.05$), Figure 4.22. The level of MDA significantly decreased ($P \leq 0.05$) in groups treated with *P. macrocarpa* and *T. crispata* compared to the vehicle control group (Figure 4.23).

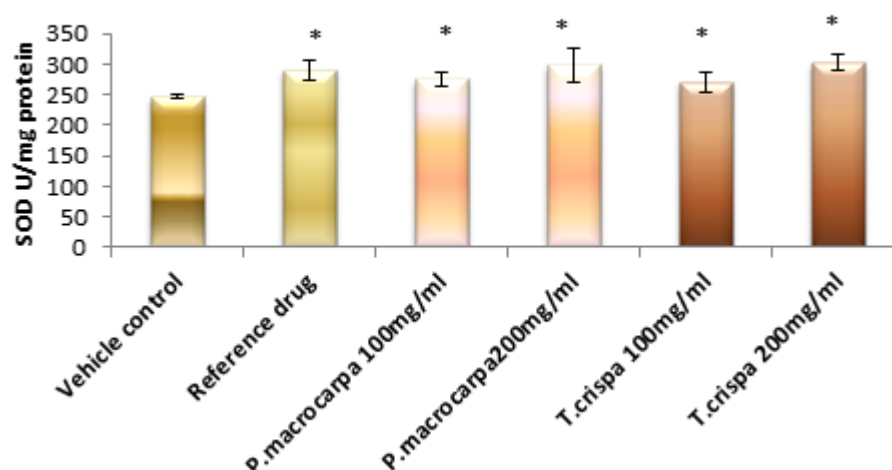


Figure 4.21: Effects of *P. macrocarpa* and *T. crispa* on SOD level in the healed wound area in the rats. Values are presented as mean \pm S.D., (5 rats/group).

* Significant ($P \leq 0.05$) compared to vehicle control

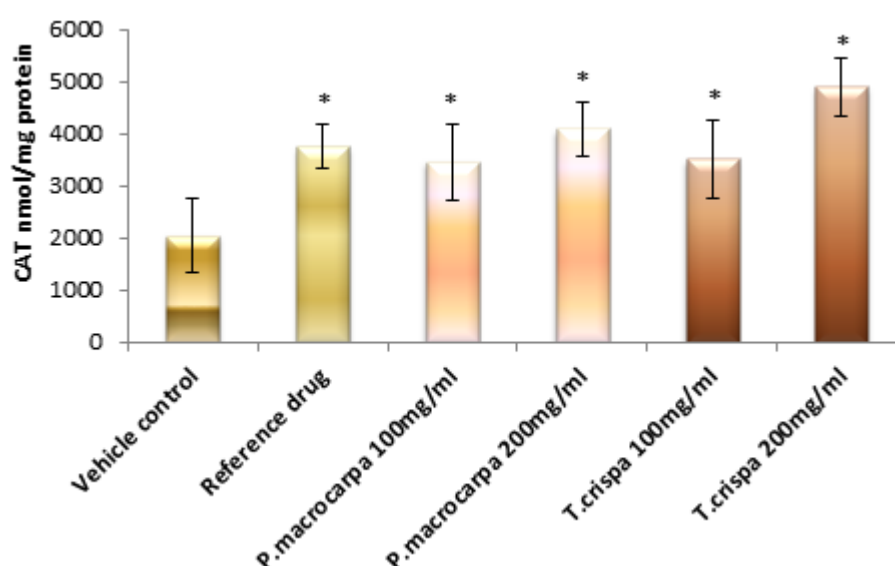


Figure 4.22: Effects of *P. macrocarpa* and *T. crispa* on CAT level in the healed wound area in the rats. Values are presented as mean \pm S.D., (5 rats/group).

*Significant ($P \leq 0.05$) compared to vehicle control

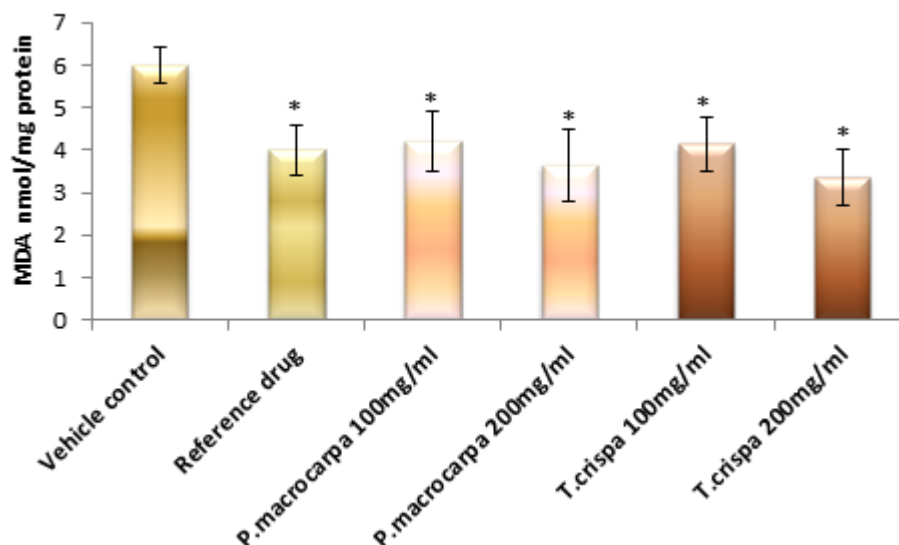


Figure 4.23: Effects of *P. macrocarpa* and *T. crispa* on MDA level in the healed wound area in the rats. Values are presented as mean \pm S.D., (5 rats/group).

* Significant ($P \leq 0.05$) compared to vehicle control

4.6.4 Estimation of TGF- β 1 and TNF- α in the wounded rat undergoing treatment

TGF β 1 and TNF- α play a role in the progress of the wound-healing process as inflammatory cytokines. Therefore, TGF β 1 and TNF- α levels in the treated groups were investigated in this study to evaluate the effect of *P. macrocarpa* and *T. crispa* on inflammatory mediators. In the group treated with two doses of *P. macrocarpa* or *T. crispa* extract, the results show that the level of TGF β 1 was significantly ($P \leq 0.05$) elevated compared to the vehicle control group and the Intracel gel treated group. The level of TNF- α was significantly ($P \leq 0.05$) lower compared to the vehicle control group (Figure 4.24 and 4.25).

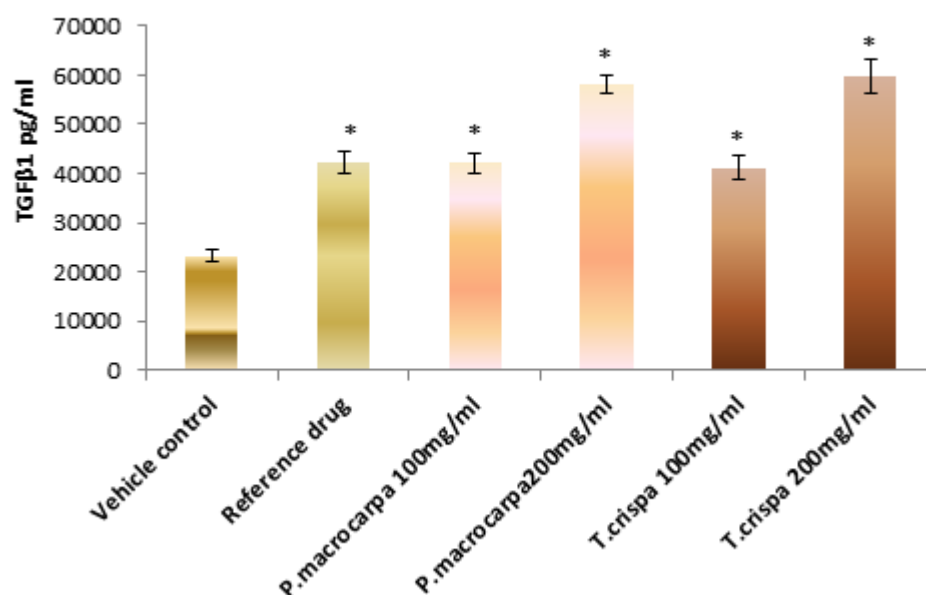


Figure 4.24: Effects of *P.macrocarpa* and *T.crispa* on TGF-β1 serum level in the treated wounded rats. Values are presented as mean ± S.D., (5 rats/group) * Significant ($P \leq 0.05$) compared to the vehicle control.

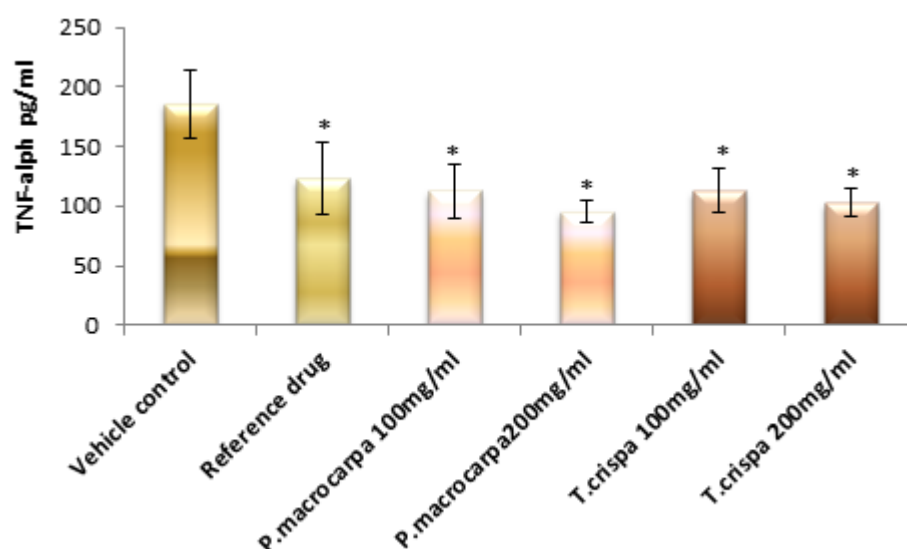


Figure 4.25: Effects of *P.macrocarpa* and *T.crispa* on TNF-α serum level in the treated wounded rats. Values are presented as mean ± S.D., (5 rats/group) * Significant ($P \leq 0.05$) compared to the vehicle control.

4.6.5 Effect on BAX and BCL2 gene expression

The results of RNA concentration and purity ($A_{260} / A_{280} \geq 1.65$) were measured by Nano Drop ND-2000 Spectrophotometer (appendix III). The results of the gel electrophoresis showed two bands of mRNA 28S and 18S that indicate the purity and integrity of the mRNA extraction (Figure 4.26).

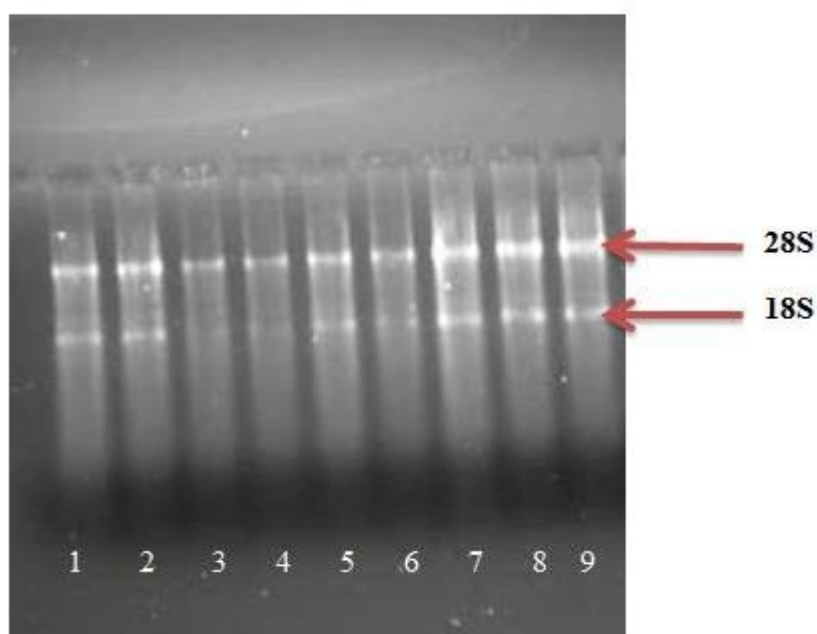


Figure 4.26: mRNA integrity profile ethidium bromide stained with agarose gel

The gene expression level of the target gene normalized with the endogenous control (HPRT1) was convergent in amplification efficiency with the target gene. All genes show according to the standard curve results an efficiency of between 90-110% (Table 4.6).

Table 4.6: Amplification efficiency and slope to the BAX, BCL2, GADPH and HPRT1 genes

Genes	BAX	BCL2	GADPH	HPRT1
Efficiency (%)	94.758	94.112	87.803	92.954
Slope	-3.454	-3.472	-3.654	-3.503

The analysis data of C_T value to the target gene and normalized with the endogenous control gene (HPRT1) showed differences in gene expression between the calibrator group (vehicle control group) and other treated groups. The relative mRNA expression level showed non-significant up-regulation of the BAX gene (Figure 4.27) in the reference drug, *P. macrocarpa* treated group at doses of (100 mg/ml) and *T. crispa* at both doses 100 mg/ml & 200 mg/ml except non-significant down-regulation happened in the *P. macrocarpa* treated group at a dose of 200 mg/ml with fold change (1.0604, 1.1933, 1.4276 and 1.0539) respectively (Table 4.7). On the other hand, the results showed non-significant down-regulation in the BCL2 gene (Figure 4.27) in all treated groups except significant ($P \leq 0.05$) down-regulation happened in *T. crispa* at a dose of 200 mg/ml (Figure 4.27) with fold change (1.1358, 1.0461, 1.3102 and 1.0412) respectively (Table 4.7).

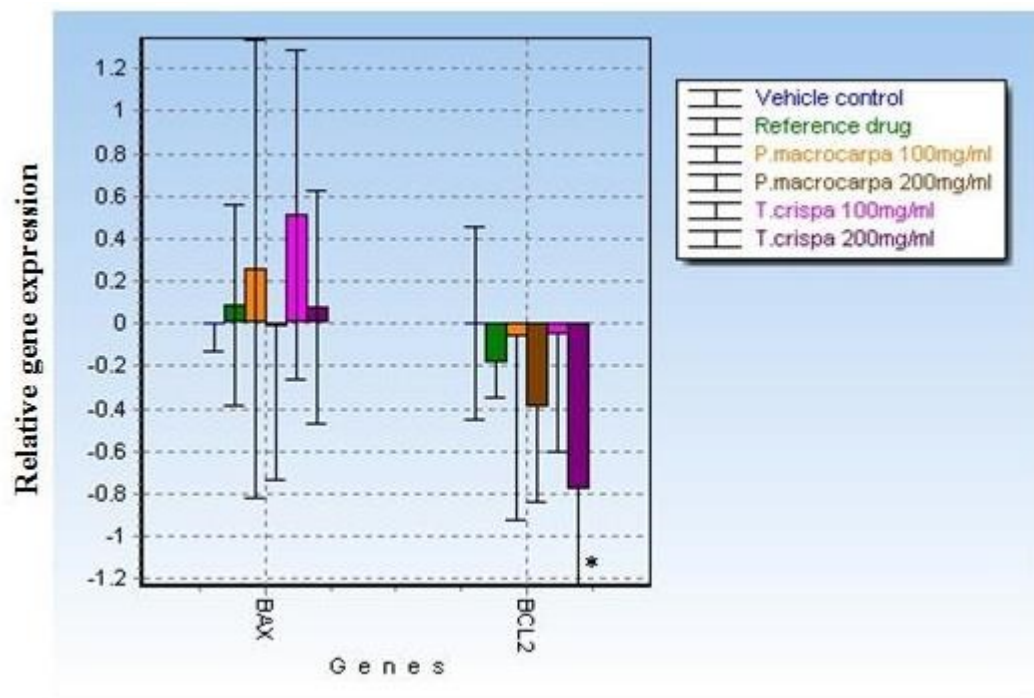


Figure 4.27: Relative gene expression levels for BAX and BCL2 genes of groups treated with *P. macrocarpa* and *T. crispa*, in comparison to the calibrator, in the skin wound. Relative gene expression levels were calculated using the delta-delta CT method and values are presented as mean \pm SD., (5 rats/group) *significantly different at $P \leq 0.05$ versus calibrator (vehicle control group).

Table 4.7: The fold change expression of the BAX and BCL2 of groups treated with *P. macrocarpa* and *T. crispa*, in comparison to the calibrator, in the skin wound.

Groups	BAX fold change	BCL2 fold change
Calibrator (Vehicle control)	0	0
Intra site (reference drug)	1.0604	1.1358
<i>P. macrocarpa</i> (100 mg/ml)	1.1933	1.0461
<i>P. macrocarpa</i> (200 mg/ml)	1.0141	1.3102
<i>T. crispa</i> (100 mg/ml)	1.4276	1.0412
<i>T. crispa</i> (200 mg/ml)	1.0539	1.7144*

* Significant ($P \leq 0.05$), (5 rats/group)

4.7 Immunomodulatory effect of *P. macrocarpa* and *T. crispa* on the RAW264.7 cell

4.7.1 Proliferation effect on the RAW264.7 cell

The crude extracts and five yield fractions of two plants were investigated for their immunomodulatory effect on the RAW264.7 macrophage cell line. Results proved that *P. macrocarpa* and *T. crispa* stimulated the proliferation of RAW264.7 cell in a dose dependent manner. The cell viability showed a significant increase ($P \leq 0.05$) with a mean viable cell percent \pm SD, (104.62 ± 3.04 , 116.35 ± 1.29 , 127.37 ± 0.96 , 136.61 ± 0.97 , 140.52 ± 4.82 , 167.54 ± 3.63 , 212.44 ± 2.23) respectively for *P. macrocarpa* at all treated doses and (126.43 ± 2.91 , 141.59 ± 1.8 , 163.62 ± 3.26 , 131.28 ± 0.65 , 127.96 ± 1.45 , 108.18 ± 1.98) respectively for *T. crispa* doses of (25, 50, 100, 200, 400 and 800) $\mu\text{g/ml}$. On the other hand, results showed no significant increase in the cell viability at doses of 1000 $\mu\text{g/ml}$ for *T. crispa* (103.79 ± 1.79) after an incubation period of 24h compared to control (Figure 4.28).

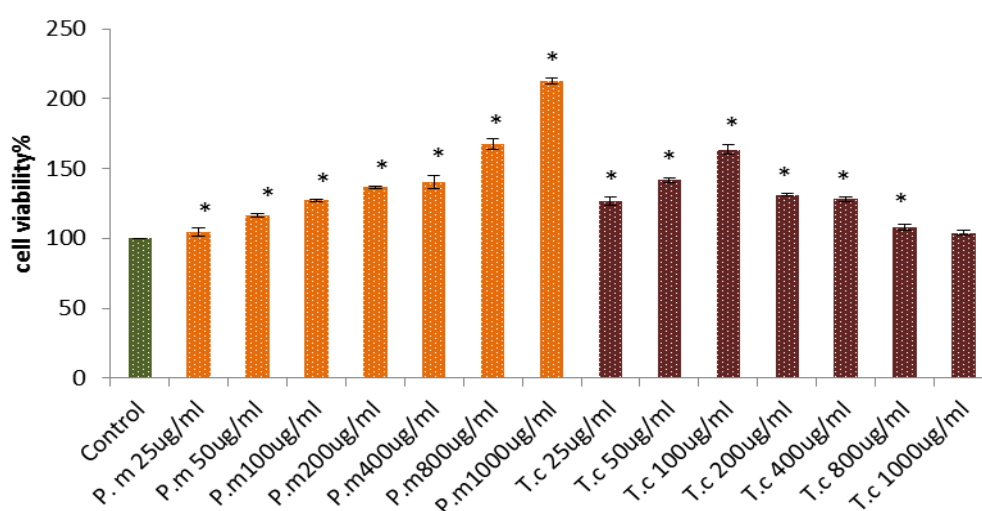


Figure 4.28: Cell viability percentage of RAW264.7 cell treated groups of *P. macrocarpa* and *T. crispa* compared to control (untreated group). Each value is presented as the mean percent \pm S.D.*significantly different versus control group, $P \leq 0.05$.

In order to research the immunomodulatory effect of the two plant fractions on the RAW264.7 cell line and choose the best one for applying in the investigation on the effect of the intracellular expression of cytokines and the subject for LCMS profiling to identify the active constituents, we investigated the effect of five fractions for both plants on the proliferation of the RAW264.7 cell line. The isolated fractions appeared to have a different effect on RAW264.7 cell proliferation. Results showed a significant ($P \leq 0.05$) increase in the viability of the cell after treatment for a 24h incubation period with *P.macrocarpa* (F1 and F5) in a dose dependent manner and a significant decrease in viability of the cell ($P \leq 0.05$) after treatment with *P.macrocarpa* (F2, F3 and F4) in a dose dependent manner (Figure 4.29).

In addition, the treatment with *T. crispa* (F1, F2, and F5) showed a significant increased viability of the cell ($P \leq 0.05$) in a dose dependent manner. On the other hand, the viability percentage showed significant decreases ($P \leq 0.05$) after treatment for a 24h incubation period with *T. crispa* (F3 and F4) in a dose dependent manner compared to the control untreated cell (Figure 4.30).

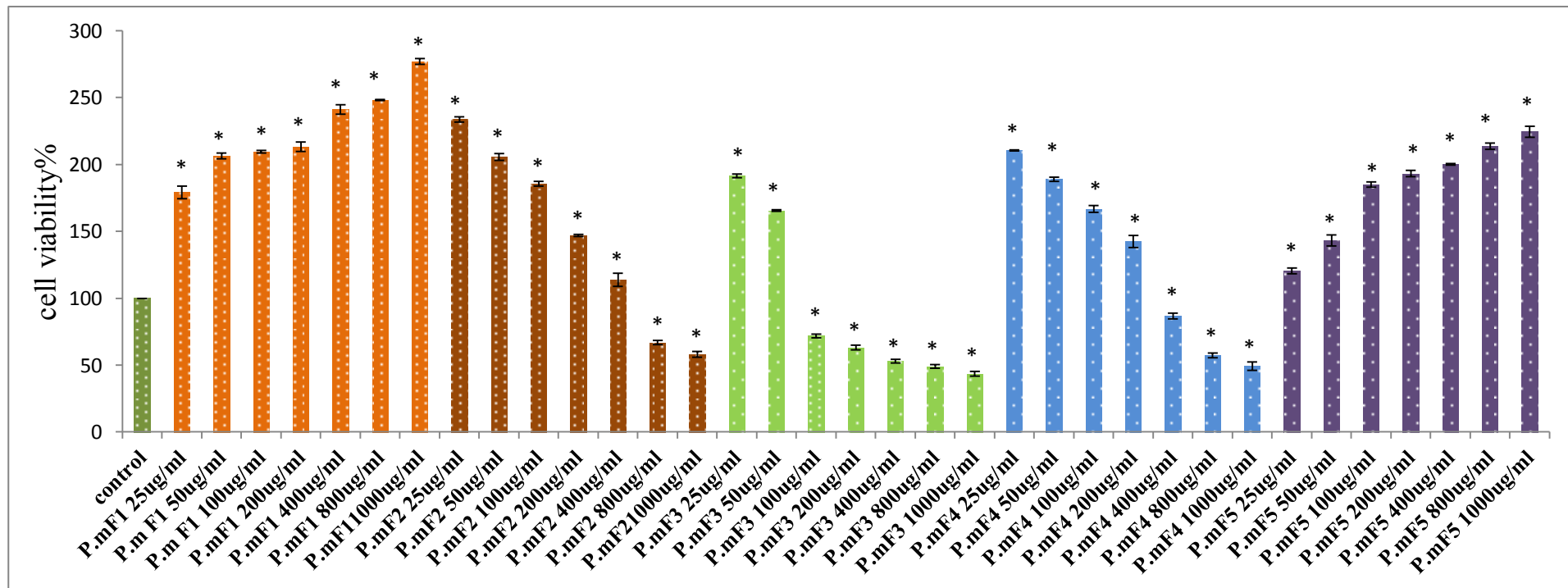


Figure 4.29: Cell viability percentage of RAW264.7 cell treated groups with *P. macrocarpa* fractions (F1, F2, F3, F4 and F5) compared to control (untreated group). Each value is presented as mean percent \pm S.D. *significantly different versus control group, ($P \leq 0.05$).

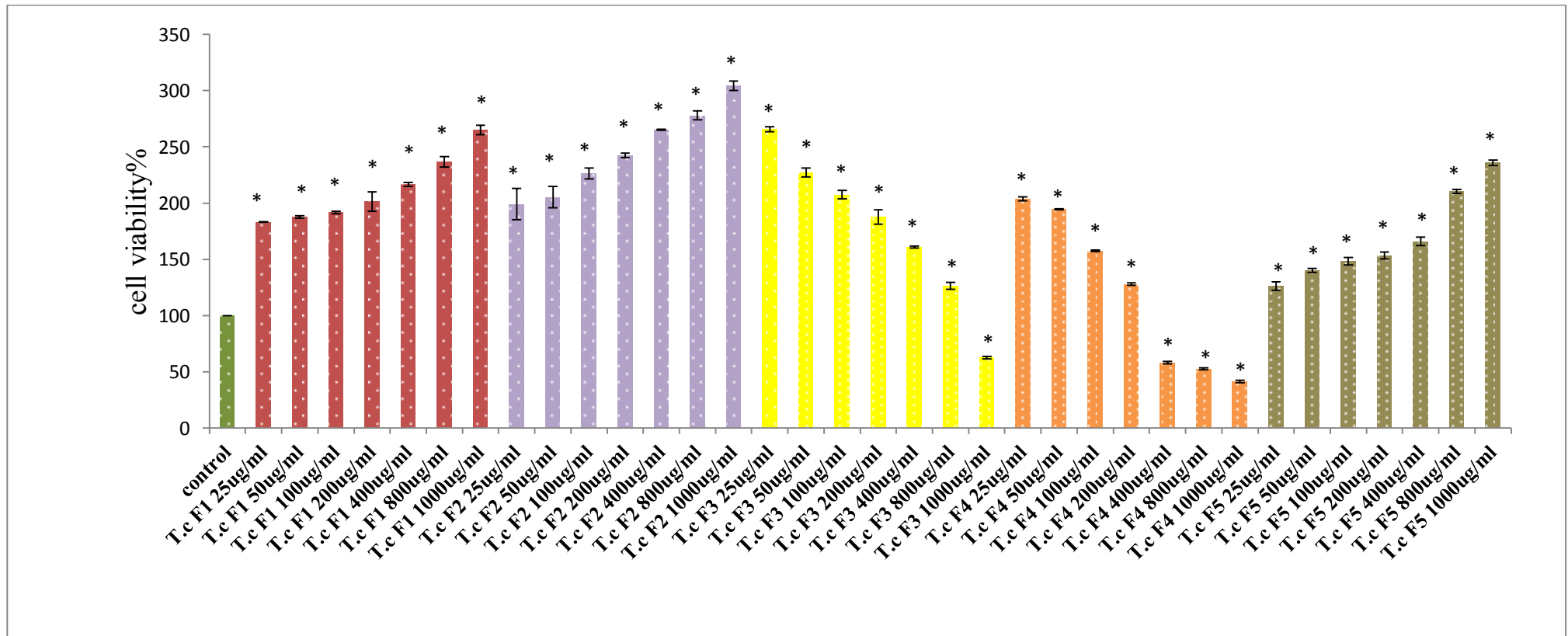


Figure 4.30: Cell viability Percentage of RAW264.7 cell treated groups with *T. crispata* fractions (F1, F2, F3, F4 and F5) compared to control (untreated group). Each value is presented as mean percent \pm S.D.*significantly different versus control group, ($P \leq 0.05$).

4.7.2 *In vitro* stimulation for intracellular cytokines production

Stimulation of the production of (IFN- γ , IL-6 and IL-8) was measured intracellularly by flow cytometry on the stimulated RAW264.7 macrophage cell with 1 μ g/ml LPS and 100 μ g/ml from each *P. macrocarpa* extract, *P. macrocarpa* F1, *T. crispa* extract and *T. crispa* F2. The intracellular expression of the IFN- γ showed significant ($P \leq 0.05$) increase in the stimulation of the RAW264.7 cell with *P. macrocarpa* extract, *P. macrocarpa* F1, *T. crispa* extract and *T. crispa* F2 at a mean percent \pm SD (25.55 ± 5.25 , 22.90 ± 5.1 , 23.15 ± 4.25 and 22.95 ± 4.35) respectively compared to control RAW264.7 cell stimulation with LPS alone (Figure 4.31).

In addition, results showed a significant increase in intracellular expression of IL-6 and IL-8 ($P \leq 0.05$) in the stimulation of the RAW264.7 cell with *P. macrocarpa* extract, *P. macrocarpa* F1, *T. crispa* extract and *T. crispa* F2. In (Figure 4.32) the results show mean percent \pm SD for IL-6 intracellular expression (14.57 ± 4.94 , 14.0 ± 4.65 , 13.67 ± 6.17 and 13.90 ± 5.53) respectively and (Figure 4.33) shows mean percent \pm SD for IL-8 intracellular expression (15.45 ± 2.75 , 14.60 ± 2.8 , 16.0 ± 3.3 and 15.9 ± 2.5) respectively compared to control (RAW264.7 cell stimulation with LPS alone).

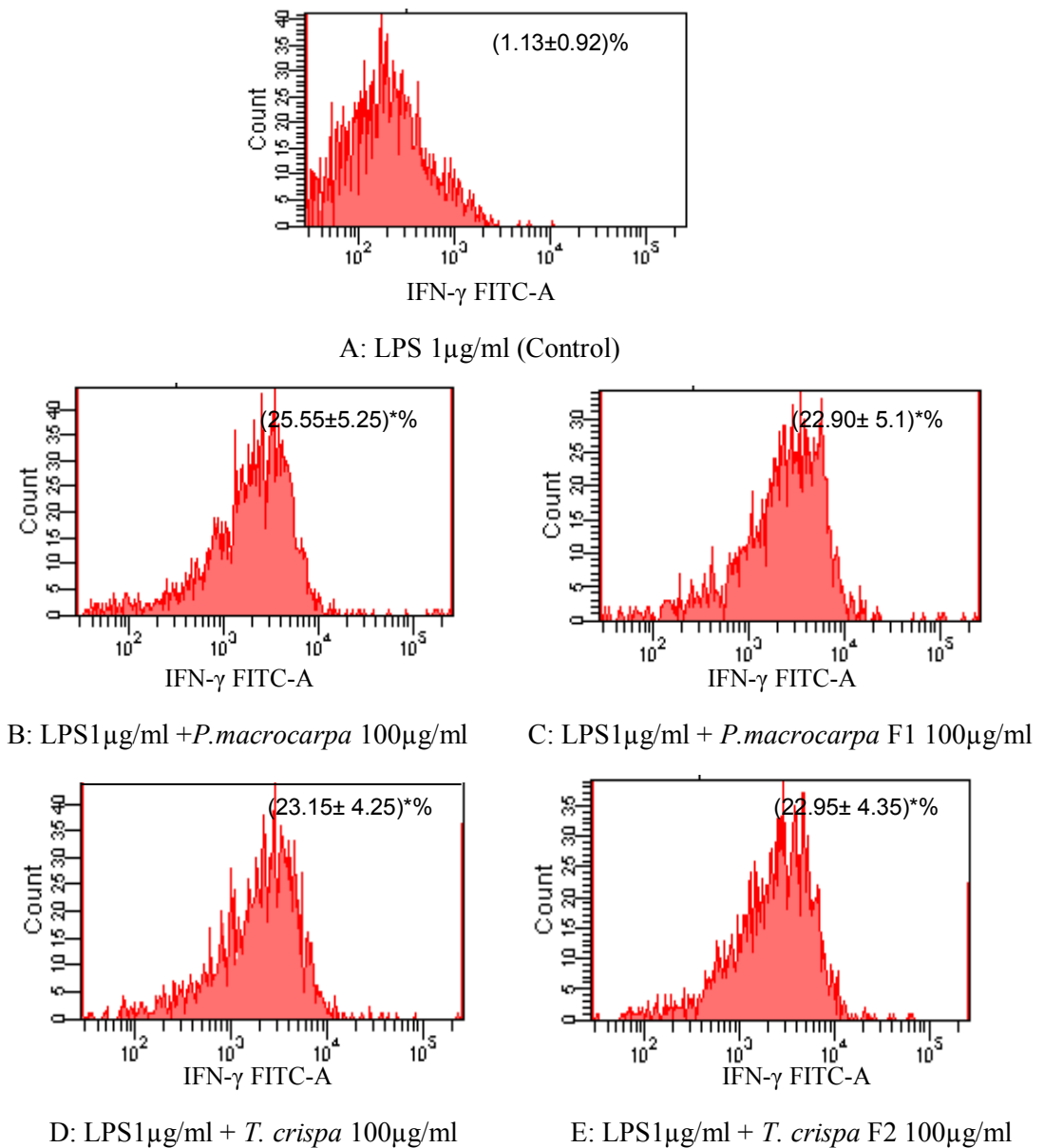


Figure 4.31: Intracellular expression of IFN- γ on RAW264.7 macrophage cell. Flow cytometry analysis was used to assess the intracellular expression of IFN- γ . The figures show the expression percent of IFN- γ on RAW264.7 cell stimulated with (A) 1 μ g/ml LPS alone as a control. (B) 1 μ g/ml LPS + *P. macrocarpa* 100 μ g/ml. (C) LPS1 μ g/ml + *P. macrocarpa* F1 100 μ g/ml. (D) LPS1 μ g/ml + *T. crispa* 100 μ g/ml. (E) LPS1 μ g/ml + *T. crispa* F2 100 μ g/ml. The number represents the mean percent of the cell \pm SD. *Significant ($P \leq 0.05$) versus control.

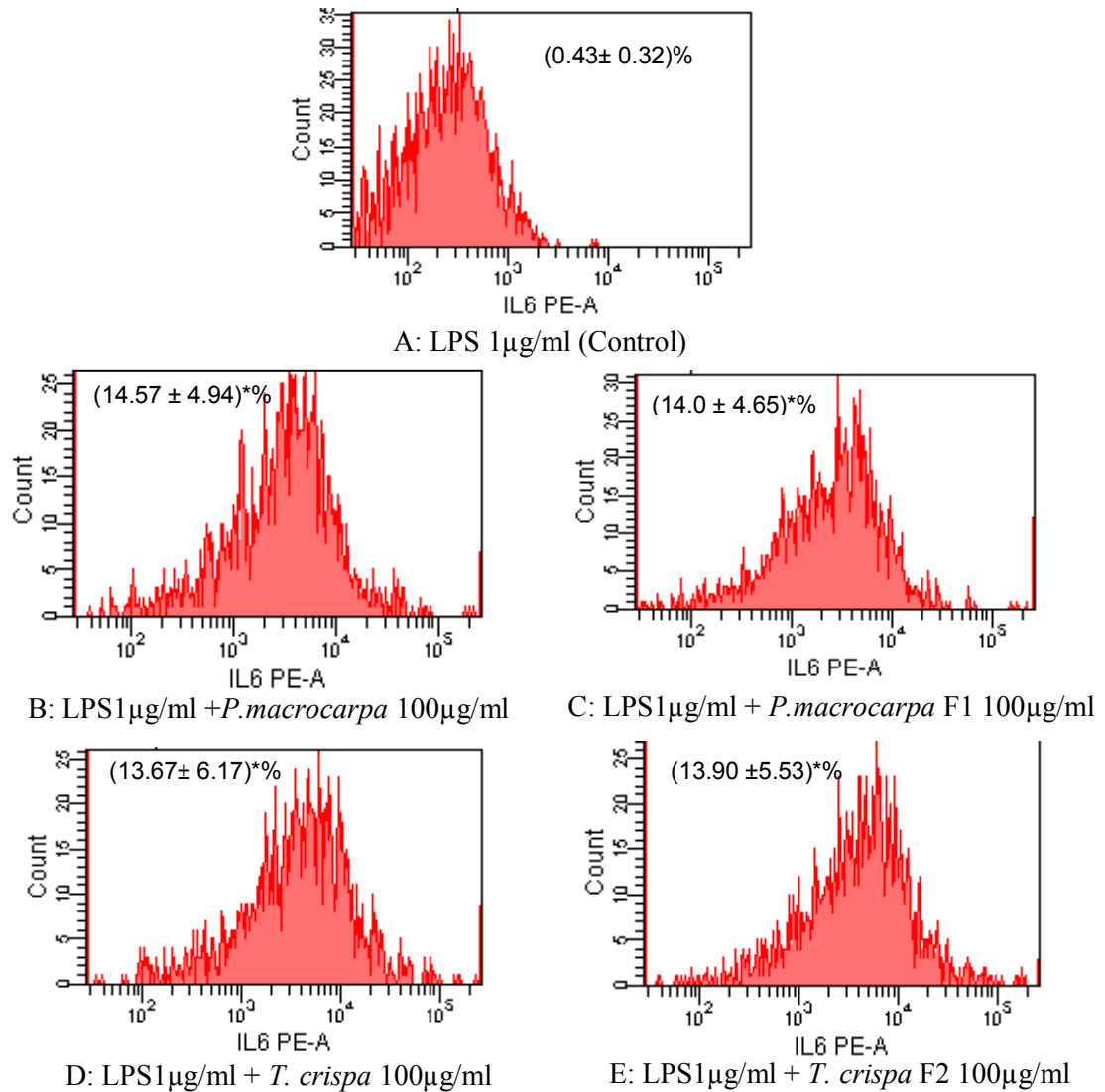


Figure 4.32: Intracellular expression of IL-6 on the RAW264.7 macrophage cell. Flow cytometry analysis was used to assess the intracellular expression of IL-6. The figures show the expression percent of IL-6 on RAW264.7 cell stimulated with (A) 1 μ g/ml LPS alone as a control. (B) 1 μ g/ml LPS + *P. macrocarpa* 100 μ g/ml. (C) LPS1 μ g/ml + *P. macrocarpa* F1 100 μ g/ml. (D) LPS1 μ g/ml + *T. crispa* 100 μ g/ml. (E) LPS1 μ g/ml + *T. crispa* F2 100 μ g/ml. The number represents the mean percent of the cell \pm SD. *Significant ($P \leq 0.05$) versus control.

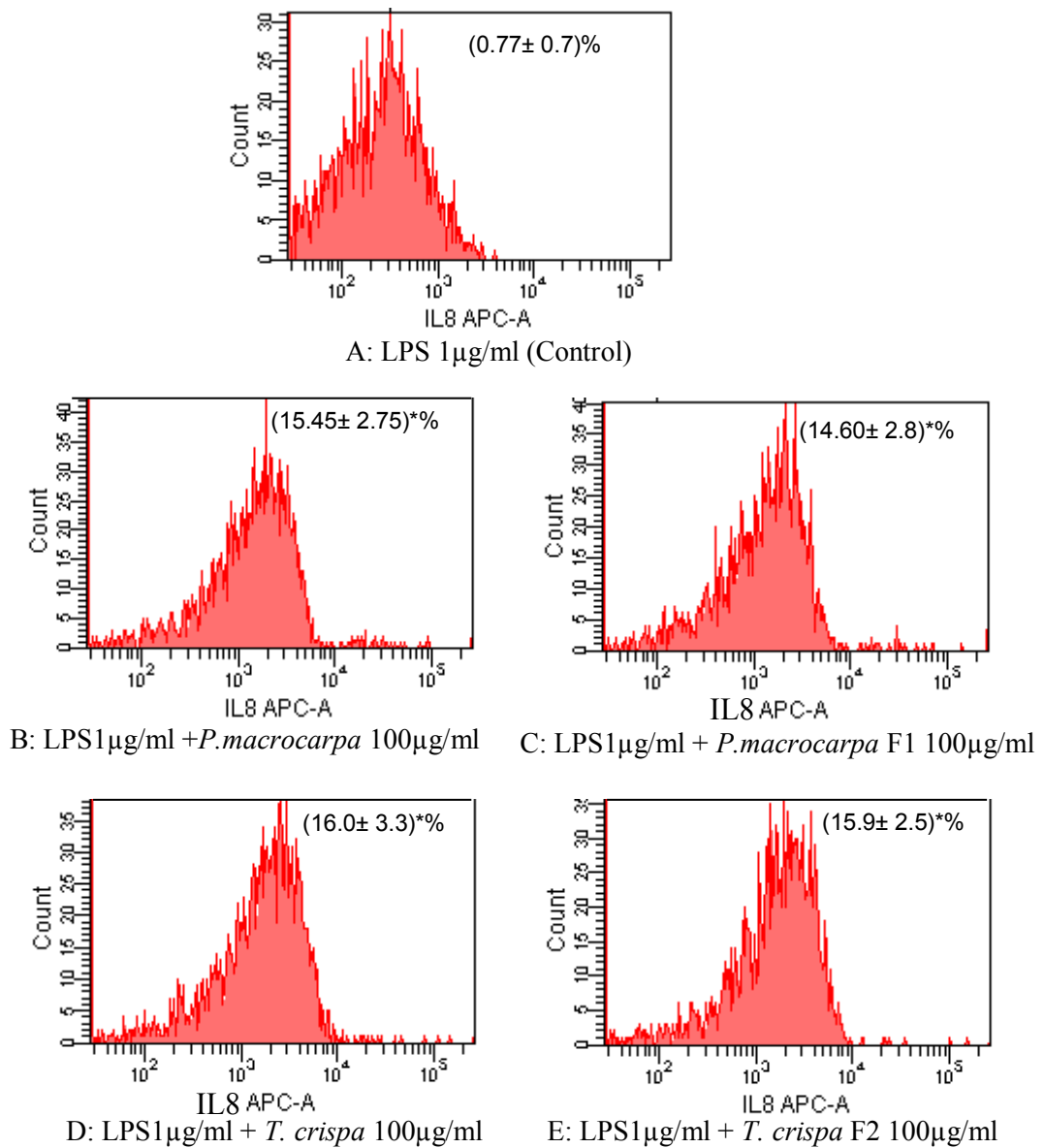


Figure 4.33: Intracellular expression of IL-8 on the RAW264.7 macrophage cell. Flow cytometry analysis was used to assess the intracellular expression of IL-8. The figures show the expression percent of IL-8 on RAW264.7 cell stimulated with (A) 1μg/ml LPS alone as a control. (B) 1μg/ml LPS + *P. macrocarpa* 100μg/ml. (C) LPS 1μg/ml + *P. macrocarpa* F1 100μg/ml. (D) LPS 1μg/ml + *T. crispa* 100μg/ml. (E) LPS 1μg/ml + *T. crispa* F2 100μg/ml. The number represents the mean percent of the cell ±SD. *Significant ($P \leq 0.05$) versus control.

4.8 Investigation of active constituents

The results of LC-MS to identify the phenolic constituents and other active compounds of *P. macrocarpa* F1 and *T. crispa* F2 fractions showed about five compounds detected for *P. macrocarpa* F1 and four compounds detected for *T. crispa* F2. Tables 4.8 and 4.9 show all peaks detected with their retention time and observed m/z and m/z of fragment ions. The *P. macrocarpa* F1 compounds contain oxoglucine at m/z 351.2518 and its fragment at m/z 236.0715 (Figure 4.34), gallic acid at m/z 171.0988 (Figure 4.35), harmaline at m/z 214.0901, its fragment at m/z 155.9790 (Figure 4.36), myricetin at m/z 318.2998 (Figure 4.37) and orotic acid at m/z 157.0183 (Figure 4.38). While *T. crispa* F2 compounds include cordioside at m/z 511.2712 (Figure 4.39), quercetin at m/z 301.1422 and its fragments at m/z 123.0913, 185.1149 and 155.1538 (Figure 4.40), eicosenoic acid (paullinic acid) at m/z 311.1457 (Figure 4.41) and boldine at m/z 327.1576 and its fragment at m/z 251.1251 (Figure 4.42).

Table 4.8: *P. macrocarpa* F1 compound identification by LC-MS data using positive ionization mode.

Peak No.	RT (min)	Interim identification	Observed m/z	m/z of fragment ions observed	Suggested formula	M.W. (g/mol)
1	0.924	Oxoglucine	351.25	236	C ₂₀ H ₁₇ NO ₅	351.35
2	1.492	Gallic acid	171.09		C ₇ H ₆ O ₅	170.12
3	6.768	Harmaline	214.09	155	C ₁₃ H ₁₄ N ₂ O	214.26
4	7.585	Myricetin	318.29	317	C ₁₅ H ₁₀ O ₈	318.23
5	14.529	Orotic acid	157.01		C ₅ H ₄ N ₂ O ₄	156.10

RT: Retention time; M.W: Molecular weight.

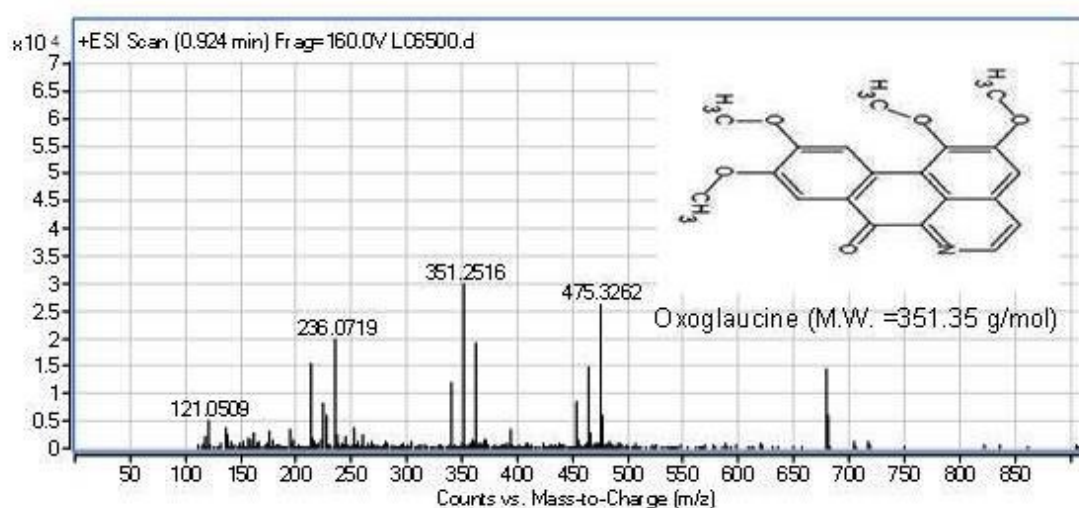


Figure 4.34: Mass spectrum (TOF MS ES+) and chemical structure of oxoglaucine (peak No. 1) identified in *P. macrocarpa* F1

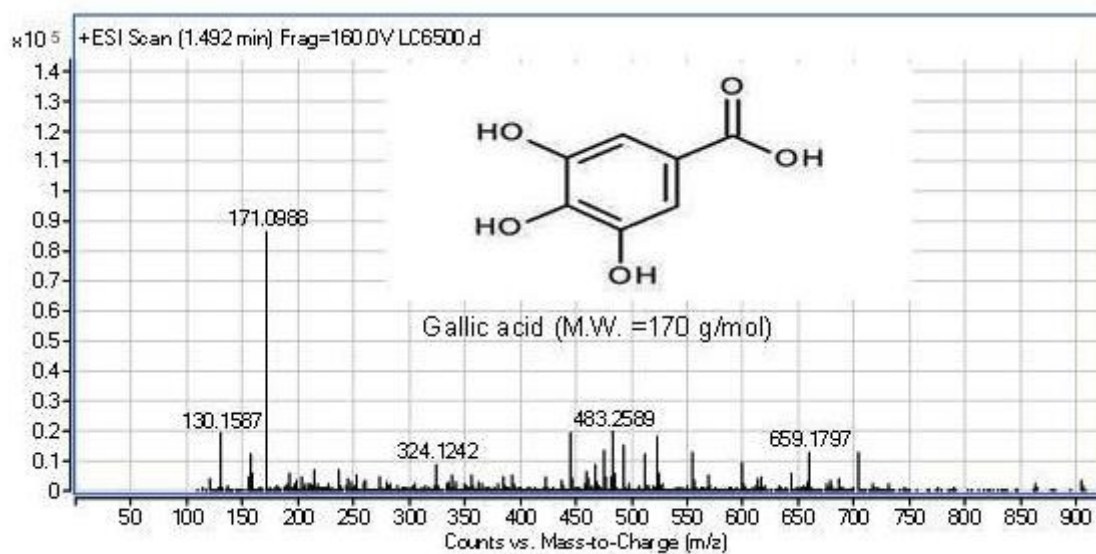


Figure 4.35: Mass spectrum (TOF MS ES+) and chemical structure of gallic acid (peak No. 2) identified in *P. macrocarpa* F1

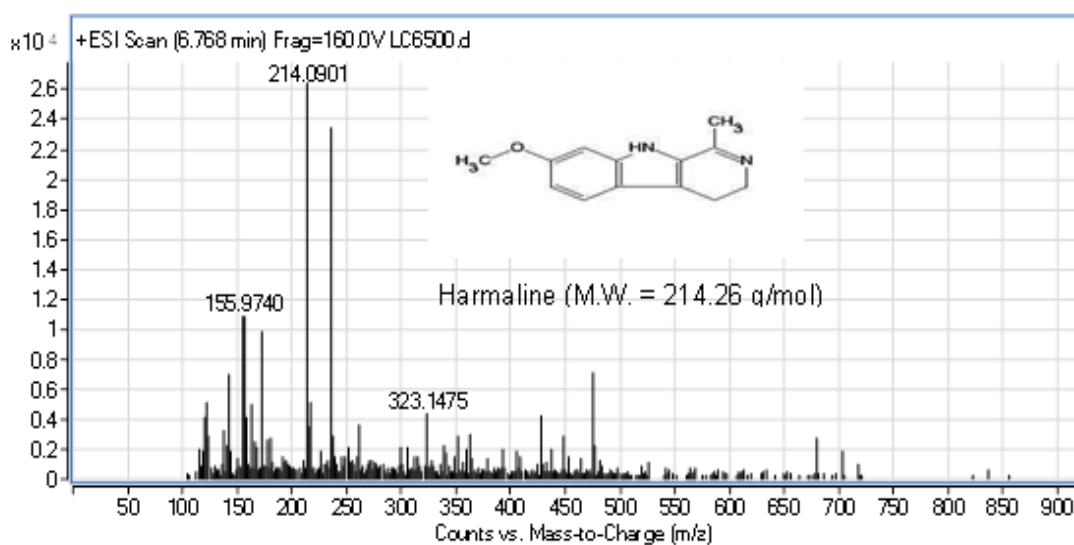


Figure 4.36: Mass spectrum (TOF MS ES+) and chemical structure of harmaline (peak No. 3) identified in *P. macrocarpa* F1

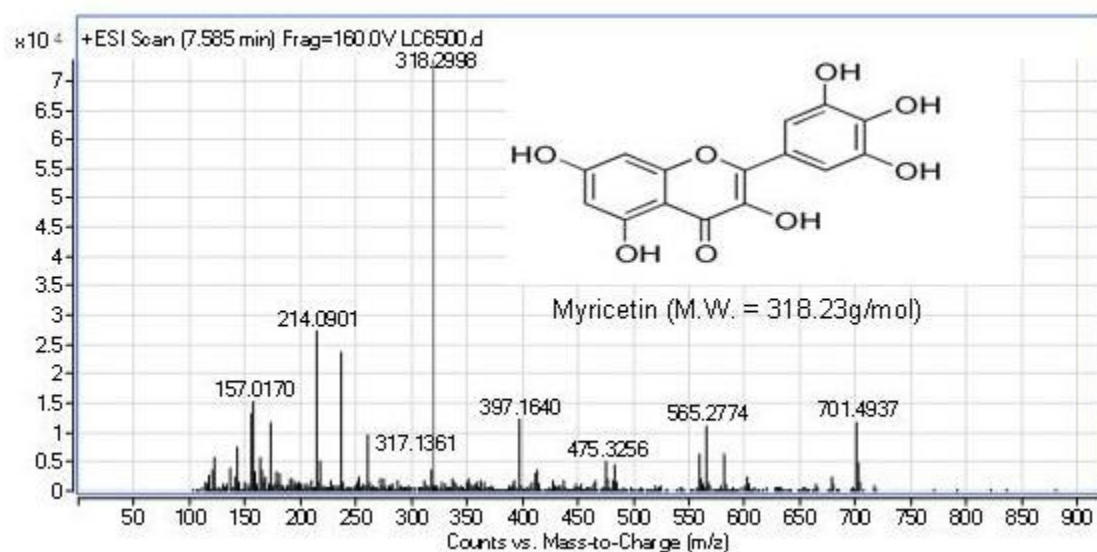


Figure 4.37: Mass spectrum (TOF MS ES+) and chemical structure of myricetin (peak No. 4) identified in *P. macrocarpa* F1

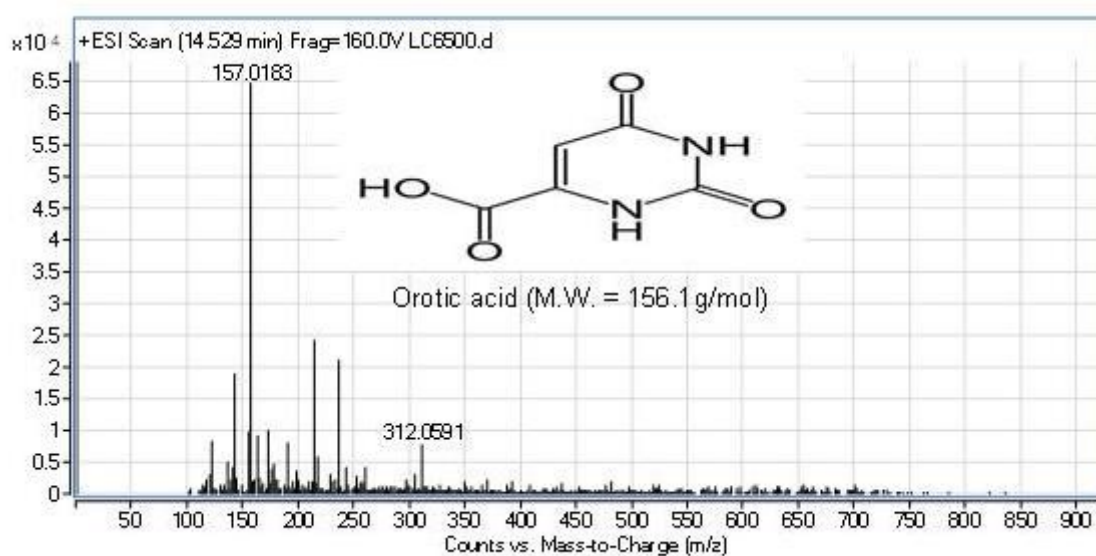
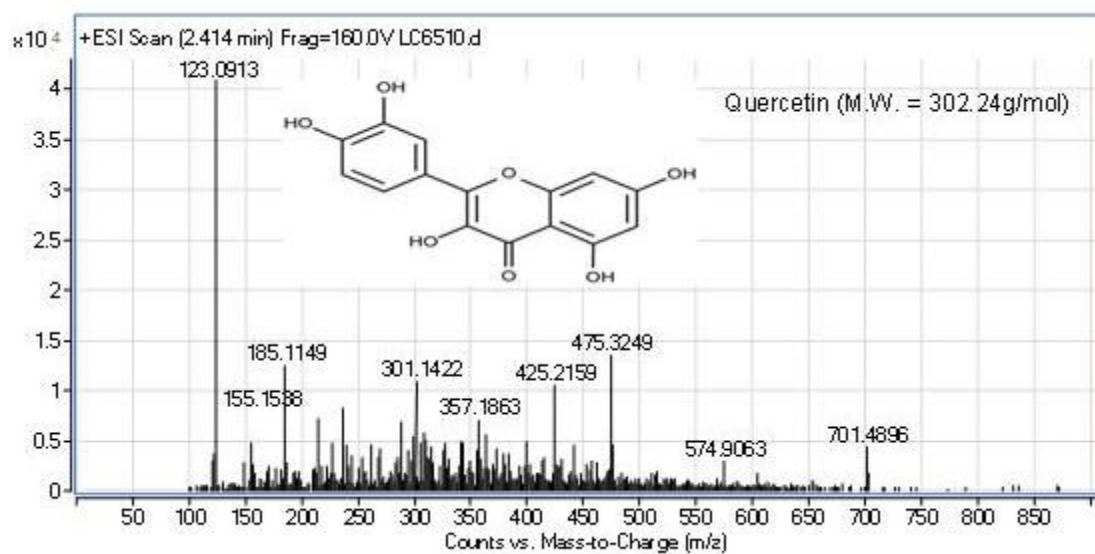
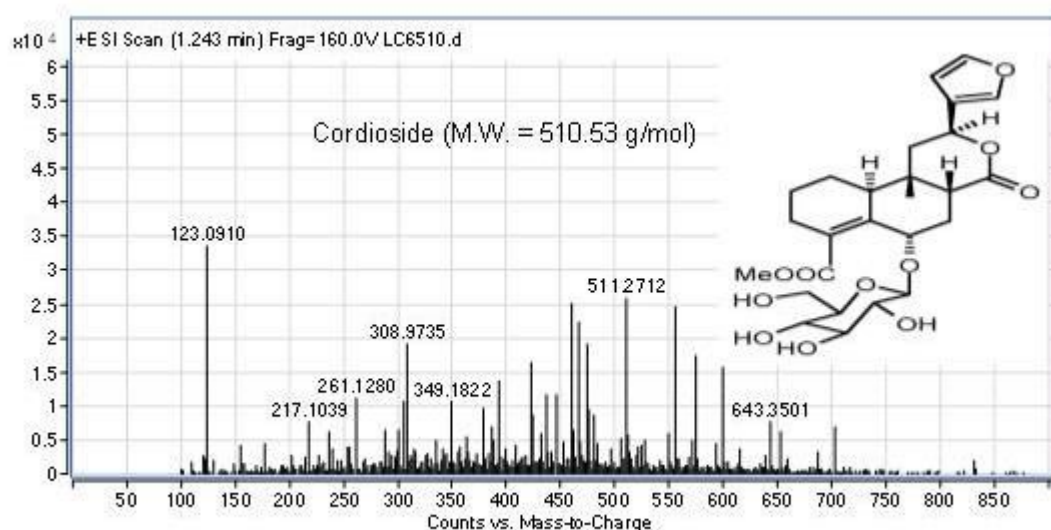


Figure 4.38: Mass spectrum (TOF MS ES+) and chemical structure of orotic acid (peak No. 5) identified in *P. macrocarpa* F1

Table 4.9: *T. crista* F2 compounds identification by LC-MS data using positive ionization mode.

Peak No.	RT (min)	Interim identification	Observed m/z	m/z of fragment ions observed	Suggested formula	M.W. (g/mol)
1	1.24	Cordioside	511.27		C ₂₅ H ₃₄ O ₁₁	510.53
2	2.41	Quercetin	301.14	123,185,155	C ₁₅ H ₁₀ O ₇	302.24
3	5.95	Eicosenoic acid (paullinic acid)	311.14		C ₂₀ H ₃₈ O ₂	310.51
4	12.42	Boldine	327.15	251.1251	C ₁₉ H ₂₁ NO ₄	327.37

RT: Retention time; M.W: Molecular weight.



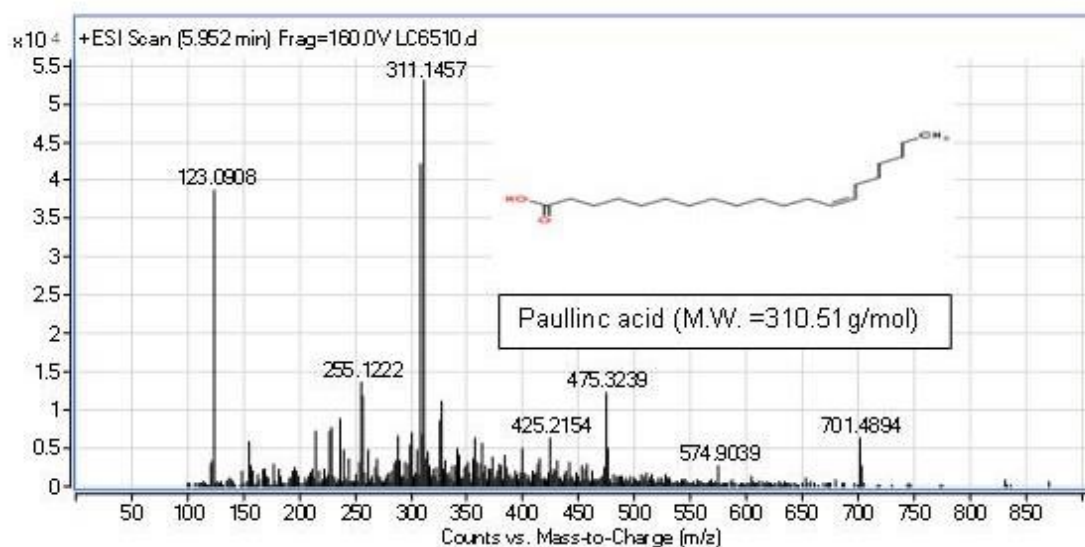


Figure 4.41: Mass spectrum (TOF MS ES+) and chemical structure of eicosenoic acid (paullinic acid) (peak No. 3) identified in *T. crista* F2

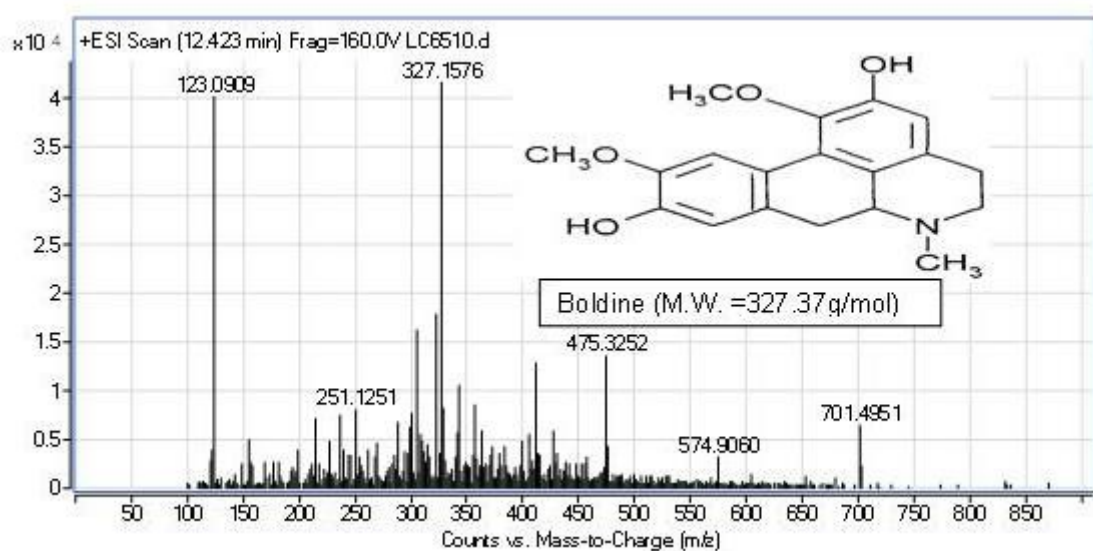


Figure 4.42: Mass spectrum (TOF MS ES+) and chemical structure of boldine (peak No. 4) identified in *T. crista* F2

CHAPTER FIVE

5 Discussion and conclusion

5.1 Discussion

5.1.1 Quantitation and evaluation of antioxidant contents and activity

P. macrocarpa and *T. crispa* were extracted according to the method described in (Sec 3.3). (13.5 g viscous dark brown was produced from 200 g powder of *P. macrocarpa*) and (34.88 g viscous dark green was produced from 200 g powder of *T. crispa*). We used ethanol as a solvent to extract antioxidant compounds such as, soluble polyphenols that are found in high concentrations in fruits and grains (Antolovich et al., 2000). However, the extracted amount of antioxidant compounds is dependent on the properties of the solvent used in extraction. Polar solvents such as water, methanol, ethanol, ethyl acetate and acetone were more suitable to extract solvents in recovering the polyphenols from the matrix of the plant (Peschel et al., 2006). Ethanol is a polar protic solvent that can be used to dissolve polar compounds and is generally used to recover antioxidant compounds from diverse parts of the plant and many different types of plants as proven by Sultana et al., 2009. The total phenolic content and total flavonoid content were extracted with higher contents (amount) and better antioxidant activities in a more polar aqueous solvent (80% ethanol , 80% methanol).

Antioxidant activity in plants has become important in recent years because of its effectiveness in general health by playing an important role in the protection of body cells from damage by oxidative stress. The secondary metabolites in plants are the most biologically active natural product compounds therefore plants are sources of food antioxidants such as vitamin E, vitamin C, phenolic acids, carotenes and phytoestrogens. Polyphenol or phenolic compounds are a group of secondary metabolites; they are composed in an optimal structure of one aromatic ring, holding one or more hydroxyl groups. The

simplest molecular form is phenolic acid and it ranges from flavonoids to more complex structure compounds (polymerized compounds) such as lignins or tannins (Calderón-Montaña et al., 2011; Gupta et al., 2012). Plants are considered a major natural source of polyphenol compounds (flavonoids and non- flavonoids), which give the color and flavor to plants (Luximon-Ramm. et al., 2002). Phenolic compounds play an important role in the life of the plant by contributing in the defense against microbial attacks. Flavonoids have also been involved in UV filtration, symbiotic nitrogen fixation and flora pigmentation. They also act as chemical messengers and regulators to physiological functions. Finally, some flavonoids act as inhibitors to growth organisms that cause disease to the plant (Chun et al., 2005).

The antioxidant activity of *P. macrocarpa* and *T. crispa* was evaluated by FRAP and DPPH tests and the results are seen in Table (4.1). *P. macrocarpa* extract has a statistically significant high antioxidant potential when compared with *T. crispa* and standard ascorbic acid. The FRAP value of *P. macrocarpa* extract is (17618.52 ± 2115.9) $\mu\text{mole Fe}^{+2}/\text{mg}$, while for *T. crispa* and the standard ascorbic acid are $(11011.11 \pm 1145.42, 7951.85 \pm 330.42)$ $\mu\text{mole Fe}^{+2}/\text{mg}$ respectively. The total antioxidant activity was assessed by the DPPH test which detected DPPH inhibition % (86.79 ± 0.4) , as well as IC₅₀ (14 $\mu\text{g}/\text{ml}$) for *P. macrocarpa*, 55.79 ± 7.9 ; IC₅₀ (22 $\mu\text{g}/\text{ml}$) for *T. crispa* and 69.03 ± 9.3 ; IC₅₀ (19 $\mu\text{g}/\text{ml}$) for ascorbic acid. The total phenolic and total flavonoid contents are displayed in table (4.1). Results reveal the total phenolic content of *P. macrocarpa* extract is $(548.51 \pm 76.51 \text{mg GAE/ g})$ of dry fruit weight and the total flavonoid content $(213.16 \pm 1.31 \text{mg QE/g})$ of dry fruit weight, while total phenolic content is $(84.598 \pm 7.82 \text{mg GAE/ g})$ and total flavonoid content $(62.07 \pm 39.76 \text{mg QE/g})$ in the *T. crispa* stem extract.

The findings of this study are in concordance with previous research on antioxidant activity of various parts of the *P. macrocarpa* (young fruit, dark fruit, seeds young, old seeds, bark, roots and leaves) (Soeksmanto et al., 2007). It was also found that the ethanol extract of young fruit has an antioxidant inhibition of 78.48% and old fruit 83.08%. Ethanol extracts of old seeds, young seeds, roots, bark and leaves have antioxidant inhibition from (38.39-44.43%). These results indicate the power of antioxidant inhibition of *P. macrocarpa* in young fruit and old fruit (Soeksmanto et al., 2007). Other studies have confirmed high contents of phenolic and flavonoid compounds in the *P. macrocarpa* fruit extract so the high antioxidant activity in the *P. macrocarpa* fruit comes from the high-level content of phenols and flavonoids (Altaf et al., 2013; Hendra et al., 2011b). Rohyami, 2008 proved the presence of flavonoid compounds, tannins and terpenoids, whereas the extract tested negative for alkaloids. (Hendra et al., 2011b) proved the presence of flavonoid compounds such as kaempferol, myricetin, naringin and rutin in the pericarp, while naringin and quercetin was in the mesocarp and the seed had only quercetin. Another study was reported isolating gallic acid from the fruit (Faried et al., 2007).

On a similar note, (Amom et al., 2011) proved that the extract of *T. crispa* stem possesses high levels of antioxidant. (Aminul et al., 2011) found that the extract of the *T. crispa* stem has strong antioxidant scavenging powers compared to ascorbic acid and BHT. Amom et al., demonstrated that the *T. crispa* stem extract has antioxidant properties resulting from the flavonoid content such as luteolin, catechin, rutin and morin, besides detecting total phenolic content (Amom et al., 2009). There was a similar result with aqueous and methanol extract showing that both extracts possess high scavenging ability and high total phenolic and flavonoid content (Ibahim et al., 2011; Ihsan et al., 2011). Many studies have revealed positive relationships between phenolic compounds

and antioxidant activity because of their ability to scavenge free radicals through their hydroxyl groups (Tosun et al., 2009). Flavonoids usually have more hydroxyl groups so they increase radical scavenging activity in the plants when they are found. Moreover, this finding was reported by many studies (Ghasemia et al., 2009; Ismail et al., 2004). Finally the findings of this study explain that the high antioxidant activity in the *P. macrocarpa* fruit and the *T. crispa* stem extract can be attributed to the high-level content of phenol and flavonoids.

5.1.2 Acute toxicity study

During the 14 days of the experimental period, there was no record of death in rats of either sex with both plants at all doses (50, 500, 2000 mg/kg). There were no toxic signs, nor were there any changes to the skin and hair, in any of the rats. The results of the acute toxicity study indicated that the LD50 of the extract *P. macrocarpa* fruit was more than 2000 mg/kg. This result confirmed a previous *in vitro* study, which showed that the ethanol extract of *P. macrocarpa* fruit was not toxic to human mononuclear peripheral normal cells (Astuti et al., 2007). An *in vivo* study on rats was performed to investigate the anti-hypercholesterolemic effect of *P. macrocarpa* fruit aqueous extract. The extract was administered orally at a dose of 40 mg/kg for 84 days and in this period no toxic sign was reported (Chong et al., 2011). On the other hand, liver enzymes were elevated with a high dose of *T. crispa* and some inflammatory cells appeared histologically in the high dose but this had no effect on the activity of the rat, and no death was reported. However, this result may be due to the side effects of the administration of the extract. Pranee et al. demonstrated that an acute toxicity study of ethanol stem extract of *T. crispa* in mice revealed that the oral dose of 4 g/kg the body weight did not cause any toxic signs. However, the chronic toxicity study of ethanol extract of *T. crispa* proposed that resulted in the renal toxic and hepato toxic potential of the extract observed in rats.

Persistent use of high doses of *T. crispa* in humans must be avoided or stopped immediately when signs of liver or renal toxicities happen while using *T. crispa* containing medicine. (Pranee et al., 1997).

According to the results of the acute toxicity study the *P. macrocarpa* fruit and *T. crispa* stem extract should be considered 'no label' based on the recommendation of the Organization for Economic Co-operation and Development (OECD): very toxic ≤ 5 mg/kg body weight; toxic $> 5 \leq 50$ mg/kg; harmful $> 50 \leq 500$ mg/kg and no label $> 500 \leq 2000$ mg/kg. The results indicated that the *P. macrocarpa* fruit and *T. crispa* stem extract should be assigned to the lowest toxicity class (Walum, 1998).

5.1.3 Gastroprotective ability of *P. macrocarpa* fruit and *T. crispa* stem

Gastric ulcer is defined as damage resulting from disturbance in the gastric mucosal defense, which means that the ulcer is caused by an imbalance between offensive and protective factors of the gastric mucosa. Pepsin and gastric acid are aggressive factors whose proteolytic effect is buffered by mucosal glycoprotein, mucin secretion, prostaglandins and cell proliferation (Schmassmann et al., 1994). Different therapeutic factors including plant extracts are used to inhibit the gastric secretion or stimulate the mucosal defense mechanism by increasing the mucus production protecting the surface epithelial cells or interfering with the prostaglandin E2 synthesis (Borrelli & Izzo, 2000). Gastro-intestinal injury is induced by various methods. This study used ethanol induced gastric ulcer which is a method widely used for induced acute gastric mucosal injury in the experimental evaluation of anti-ulcer activity (Abdulla et al., 2010; Mei et al., 2012).

The injury of ethanol-induced ulcer is dominant in the glandular part of the stomach. Administration of absolute ethanol orally in rats is destructive to the stomach and, affects the gastric mucosa by disabling its barrier and exciting microvascular changes a few minutes after its application. A strong and rapid vasoconstriction in combination

with vigorous and rapid arteriolar dilation causes induction damage in mucosal capillaries (Glavin & Szabo, 1992; Ko et al., 1994). In a previous study it was reported that damage of the rat gastric mucosa induced by ethanol stimulates the formation of leukotriene C₄ (LTC₄) (Peskar et al., 1988), mast cell secretory products and reactive oxygen species (Rao et al., 2004). The necrotic lesion of the gastric mucosa is caused by ethanol and happens through multiple factors. Ethanol causes a disturbance to the barrier of the mucus bicarbonate when it reaches the mucosa and results in the rupture of the cell in the wall of blood vessels. These effects are caused perhaps by biological activities, such as formation of free radicals, peroxidation of the lipid, intracellular oxidative stress which affect the penetrability and depolarization of the mitochondrial membrane before cell death (Sannomiya et al., 2005). In addition, ethanol rapidly penetrates into the gastric mucosa and produces linear hemorrhagic lesions, damaged areas in the deep layers of the mucosa with necrosis, inflammatory cell penetration, loss of epithelial cells in the stomach and cellular apoptosis that are classic features of alcohol damage (Li et al., 2013b; Luo et al., 2013). Various mediators such as lipoxygenase, cytokines and oxygen derived free radicals have an effect in the pathogenesis of ethanol induced gastric mucosal damage directly and indirectly (Abdel-Salam et al., 2001). Disturbances in gastric secretion, damage to gastric mucosa, alterations in permeability, gastric mucus depletion and free radical production are reported to be the pathogenic effects of ethanol. Oxygen derived free radicals have been implicated in the mechanisms of acute ulceration, and scavenging of these free radicals can play a crucial role in gastroprotection and the healing of ulcers. Oral administration of ethanol causes severe gastric mucosal damage and significantly increases lipid peroxidation levels and decreases antioxidant enzymes (Gupta et al., 2005). Proton pump inhibitors (PPI) are the most potent medication available to inhibit gastric acid secretion. Since their introduction in the late 1980s, they have

been used as acid suppressants and to treat acid peptic disorders (Ali et al., 2009). They represent a group of drugs that inhibit H^+/K^+ ATPase, the enzyme known as “proton pump”, found in the luminal compartment of the parietal cell (Meneghelli et al., 2000). Omeprazole, a proton pump inhibitor, is known to function not only as a PPI but also as an anti-inflammatory agent, an antioxidant or a stimulator of gastric mucus secretion, and is used in gastric ulcers and other acid dyspeptic disorders of the upper gastrointestinal tract in a dose of 20-40 mg orally once daily. PPI commonly has a few different effects, the most common side effects are nausea, headache, constipation, abdominal pain, flatulence and diarrhea. These side effects are generally mild, self-limiting and unrelated to dosage and age. However, long-term side effects of PPI have recently gained attention and many studies have been looking at various side effects that may be related to long term use of PPI (Ali et al., 2009). In our study, we used omeprazole as the reference drug to compare the effect of *P. macrocarpa* and *T. crista* on gastroprotection.

Currently, the world trend is to use the plants and their derivative compounds in gastroprotection in order to try a natural source medication and to prevent and treat gastric disorders. Many studies have been done to investigate the gastroprotection of different plants in a number of countries. For example, *Cymbopogon citratus* is a medicinal plant used to treat different diseases, and the gastroprotective potential of its essential oil has been investigated and proven to reduce gastric damage induced by ethanol (Fernandes et al., 2012).

Similarly, *Rhizophora mangle* has long been used traditionally as an anti-ulcer treatment (de-Faria et al., 2012). *Piper tuberculatum* is reported to have gastroprotective effect through increased gastric mucus production and reduction of gastric acid (Moura Burci et al., 2013). *Citrus lemon* has gastroprotective properties against ethanol induced

gastric ulcer, the protection being through the involvement of a different mechanism (Rozza et al., 2011). In this study, *P. macrocarpa* and *T. crispa* were used to investigate their ability in gastric protection. The pretreated groups with different doses of *P. macrocarpa* fruit or *T. crispa* stem extract showed significantly decreased ulcer area and increased ulcer inhibition percentage (Figure 3.4) by increasing the production of mucin from mucous cells to form the first line of defense to protect the mucosal epithelial layer from damage by the ulcerogenic action of ethanol. Mucus is an important protective factor of the gastric mucosa. It presents itself as a transparent gel formed by water and glycoproteins which covers the gastrointestinal mucosa and protects the gastric mucosa against irritant agents such as ethanol and HCL (Shirazi et al., 2000). Results of the mucus barrier content analysis disclosed that the quantity of mucus is greater than normal when pretreated with the *P. macrocarpa* fruit or *T. crispa* stem extract. This suggests a cytoprotective action of the two plant extracts. Additionally, the anti-secretory activity of *P. macrocarpa* fruit or *T. crispa* stem extract might be important in the protection of the gastric mucosa. The cytoprotective effect was confirmed by histological examination showing the prevention of mucosal hyperemia and edema. The observed results showed that *P. macrocarpa* fruit or *T. crispa* stem extract protects the gastric mucosa, this protection is due to the inhibition of gastric acid secretion and rising stomach pH (Figure 4.4) by the extract. The anti-secretory activity of *P. macrocarpa* fruit and *T. crispa* stem extract were investigated by measuring stomach pH. The obtained data revealed that there was an increase of stomach pH compared to the vehicle control group and ulcer control group. Thus, the mechanism of mucosa protection may be the anti-secretory activity of *P. macrocarpa* fruit and *T. crispa* stem extract and its effect on enhancing mucin production. Similar results were observed in previous studies, *Zanthoxylum rhoifolium* showed gastroprotective ability against erosion ethanol by significantly

increasing mucus production (Freitas et al., 2011). *Indigofera suffruticosa* inhibited gastric mucosal caused by ethanol by significantly increasing the PGE2 and mucus secretion (Luiz-Ferreira et al., 2011).

To provide supplementary evidence, we investigated the effect of *P. macrocarpa* fruit and *T. crispa* stem extract on the level of PGE2 in pretreated groups. This study clearly showed a significantly elevated level of PGE2 in the pretreated groups (Figure 4.9) compared to the ulcer control group. Therefore, this result clearly indicates the involvement of PGE2 in the gastroprotective mechanism. It is known that the protective mechanism exhibited for mucus and bicarbonate secretion, depends on PGE2 secretion and this in turn depends on the activity of the cyclooxygenase (COX) enzyme system (Dey et al., 2006). In the stomach, prostaglandins promote mucus and bicarbonate secretion, maintain the normal response of the gastric environment to intrinsic factors and inhibit the inflammatory mediator release from mast cells and free radical production. Increased changes in mucosal blood flow around the ulcer in experimental animals (Szabo & Tarnawski, 2000) similarly suggest that there is an increase in the concentration of PGE2 in ulcerative regions, compared to other parts of the gastric mucosa, since PGE2 causes vasodilation. The large blood supply seems to reflect the active re-epithelization which requires an abundant supply of glucose and oxygen (Zhang et al., 2010). The findings of this study are supported by the results obtained through investigating the effect of *Decalepis hamiltonii* root polysaccharide as an anti-ulcer agent against ethanol induced gastric ulcer. The cytoprotective is increased due to the enhanced synthesis of PGE2 which is necessary in the proliferation of gastric mucin cells (Srikanta et al., 2010).

Similar results have been seen in previous studies, through studying the effects of plant extracts as gastroprotective drugs (de-Faria et al., 2012; Hiruma-Lima et al., 2002;

Megala & Geetha, 2012; Moraes et al., 2009; Rozza et al., 2011). Therefore, the increasing PGE₂ production obtained in the pretreatment with *P. macrocarpa* fruit and *T. crispa* stem extract clearly indicates the stimulation of cytoprotective factors. *P. macrocarpa* fruit and *T. crispa* stem extracts have prevented ethanol from depleting the gastric wall mucus. Prostaglandins or sulfhydryl compounds contribute to protecting the stomach from ethanol injury. A copious amount of gastric mucus is secreted during superficial mucosal damage and provides a favorable microenvironment for repair by restitution. Therefore, it is conceivable that the protection from gastric ulcers observed with both the plants that are included in this study provide general evidence of the close relationship between these factors.

To understand the significance of the gastroprotective ability results of *P. macrocarpa* fruit and *T. crispa* stem extract, the role of gastrin and pepsin in the gastroprotection effect of both plants needs to be investigated. Gastrin is a polypeptide gastrointestinal hormone with an important role in modulating various functions of the gastrointestinal system. The two major roles are the stimulation of gastric acid secretion and cell proliferation. Gastrin levels in plasma, gastric juice and the antral mucosa tissue are increased in rats with ulcer (Shulkes & Baldwin, 2013). The principle stimulant of the gastric acid secretion through stimulating gastrin receptors on the enterochromaffin like cell (ECL) in the oxyntic mucosa is gastrin secreted by antral G cells, which causes accelerated synthesis and release of histamine, which in turn stimulates H₂ receptors on adjacent parietal cells and activates acid secretion. Therefore, gastrin primarily controls acid secretion by the control of parietal cells and ECL cells. While the lack of gastric acid does not appear to effect digestion, gastric acid is important for maintaining gastric homeostasis. Gastrin acts through the cholecystokinin 2 receptors (CCK₂ receptors). The CCK₂ receptor has a stimulatory effect on gastric acid secretion

through the direct effect of CCK2 receptors on the parietal cells (Shulkes & Baldwin, 2013). Numerous histological effects of gastrin are mediated via CCK2 receptors. Studies with knockout mouse models (gastrin and CCK2 receptor knockout) and transgenic gastrin over-expression mice have supplied supplementary ideas on the role of gastrin. A decrease in acid secretion, parietal cell number and inhibition in functional activity of the histamine-ECL cell pathway in mice have been shown (Friis-Hansen, 2007).

The results of the current study show that pretreating rats with *P. macrocarpa* fruit and *T. crispa* stem extract induced a marked decrease in the serum gastrin level compared to the ulcer control group (Figure 4.7). This result indicates the action of both plants in more than one antiulcer mechanism. Gastric secretion was decreased after extract treatment and this may be a negative feedback mechanism in which an increase in PGE₂, mucus reduces the gastrin released in rats pretreated with *P. macrocarpa* fruit and *T. crispa* stem extract, thus providing a protective environment against injury caused by ethanol. In addition, this may be associated with a lack of CCK2 receptor activation and thus there was no more gastrin production. Peptides of the gastrin-CCK family act on the comparatively well characterized cholecystokinin (CCK2, gastrin CCKB and CCK1) receptors. Gastrin has a high affinity for CCK2, but not for the CCK1 receptor. Gastrin plasma concentrations are normally about 5 to 10 times higher than those of CCK after a meal. Therefore gastrin is probably a major physiological agonist of CCK2 receptors. The latter are present on the parietal cells, ECL cells and some smooth muscle cells as well as in the pancreas and brain. Experimental research suggests that there is an increase in the CCK2 receptor expression response to tissue injury or damage. This may be related to increased cell proliferation as part of the tissue repair mechanism (Dockray, 2004).

Increased levels of gastrin were found in the ulcer control group that were related to inducing the proliferation of cells as an important step in the tissue repair mechanism. This result was confirmed by the significant decrease in the level of pepsin in the pre-treated groups compared to the ulcer control group as a result of the decreased gastrin level and elevation of stomach pH. This in turn, caused inhibition of the activation of pepsinogen to pepsin so that the results in this study show a decrease in the serum pepsin level. Pepsin is an enzyme in the stomach responsible for the digestion of proteins by processing them into smaller pieces. Pepsin is excreted in the inactive form of pepsinogen in the stomach from the chief cells that release pepsinogen. To be activated pepsinogen needs a low stomach pH and hydrochloric acid (HCL) for transformation to pepsin. HCL is also produced by the gastric mucosa that releases parietal cells in the stomach lining. Release of both pepsinogen and HCL from the stomach lining is triggered by the hormone gastrin and the vagus nerve when food is ingested. HCL creates an acidic environment and is necessary to convert the inactive enzyme and to maintain the optimum acidity (pH 1-3) for pepsin function. If the pH is more than 5.0 then pepsin will denature and become inactive (Tang, 2013). The findings showed a decrease in the pepsin level in the groups pretreated with *P.macropcarpa* fruit and *T. crispa* stem extract compared to the ulcer control group (Figure 4.8). In groups pretreated with plant extract, there was a decrease in the gastrin level and a rise in the stomach pH that affected the release of pepsinogen and HCL from the stomach cells and converted pepsinogen to an active form pepsin. Therefore, any reduction in the proteolytic ability of this broad-spectrum enzyme will reduce the damage done to these mucosal surfaces. Indeed, previous studies have shown that raising the stomach pH negates the activity of pepsin (Aihara et al., 2003) and decreasing the stomach pH with accompanying elevations of

pepsin level increases the risk of producing ulcer lesions (Kotani et al., 2007; Tobey et al., 2001).

To prove the gastroprotective ability of both the plants included in this study we investigated their effect on the activity of antioxidant enzymes and the ability of both plants to scavenge the free radicals resulting from the oxidative stress induced by ethanol. Reactive oxygen species (ROS), such as hydroxyl radical and superoxide radical anion, are considered major causative factors for mucosal lesions through oxidative stress. ROS has a main role in tissue injury through the pathogenesis of several disorders of the digestive tract (Casa et al., 2000). Lipid peroxidation (LPO) is an important indicator of hydroxyl radical induced oxidative damage of the membranes and has a critical role in the pathogenesis of gastric ulceration. Ethanol increases free radical generation and decreases endogenous GSH production (Glavin & Szabo, 1992). Endogenous antioxidant enzymes, such as SOD and CAT, scavenge these ROS and prevent LPO and tissue damage. Thus in pathological conditions like gastric ulceration, ROS may be produced in excess and disrupt the delicate balance between ROS and endogenous antioxidants or antioxidant enzymes (Bhattacharya et al., 2006). Tissue contains various endogenous antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT) which scavenge these ROS and therefore prevent tissue damage.

In such situations, *P. macrocarpa* and *T. crispa* can augment the activity of ROS scavenging enzymes, prevent LPO by scavenging ROS, decrease the offensive effect of ROS and decrease LPO in pretreated groups. Therefore, inhibiting LPO and increasing free radical-scavenging antioxidant enzymes SOD and CAT (Figure 4.10 & 4.11) may also be involved in the beneficial effect of *P. macrocarpa* and *T. crispa* in preventing gastric ulceration. This scavenging ability of *P. macrocarpa* and *T. crispa* comes from their high phenolic and flavonoid contents. The latter, which has been proven in many

previous studies as a natural antioxidant source, has the ability to scavenge ROS and prevent the oxidative stress which causes damage to cells (Barros et al., 2010; Li et al., 2013a; Suhartono et al., 2012; Tawde et al., 2012). In similar research numerous medicinal plants have been employed for their antioxidant potential and investigated for their gastroprotective abilities (Hariprasath et al., 2012; Orsi et al., 2012; Viana et al., 2013).

One of the important findings in this study was that the level of pro-inflammatory factor TNF- α significantly decreased (Figure 4.14) in groups pretreated with *P. macrocarpa* and *T. crispa* extract, which increased the level of anti-inflammatory cytokine TGF- β 1 (Figure 4.13). These results indicate that the immunomodulatory effect of *P. macrocarpa* and *T. crispa* might be affected through the inflammatory mediator. Gastrointestinal mucosa integrity depends on the balance between defensive and offensive factors and many of the components of mucosal defense have proven to be influenced by inflammatory mediators, such as prostaglandins, leukotrienes, and thromboxanes (Wallace & Chin, 1997). Prostaglandins can also have anti-inflammatory effects through the inhibition of leukocyte recruitment which can contribute to their beneficial effects during situations such as inflamed gastrointestinal mucosa (Robert et al., 1985). TNF- α is a key mediator of intestinal injury caused by endotoxins and one of the mechanisms through which TNF- α can develop inflammatory responses; tissue injury is via its regulation of the expression of receptors for other inflammatory mediators, including leukotriene B4 and platelet activating factor (Sun & Hsueh, 1988).

Prostaglandins are powerful inhibitors of TNF- α released from both the macrophage (Kunkel et al., 1988) and the mast cell (Wallace & Chin, 1997), which may cause elevation of PGE2 levels during pretreatment with *P. macrocarpa* and *T. crispa*. In turn, this causes inhibition of the production of TNF- α . Another immunomodulatory effect of

P. macrocarpa and *T. crista* is by inciting the production of TGF- β 1, an important cytokine that accelerates gastric ulcer healing. The healing of gastric ulcer is a complex process controlled by numerous factors, hormones, and cytokines. TGF- β 1 is one of the multifunctional peptide growth factors that exhibit positive, regulatory healing of gastric ulcer by inducing cell migration and angiogenesis and improving extracellular matrix production. TGF- β 1 exerts its action by binding to its transmembrane serine/threonine kinase receptor, which in turn triggers activation of various intracellular signaling pathways (Tanigawa et al., 2005). A previous study has reported that the immunoreactive effect of TGF- β 1 protein is focused on the epithelial cells and, thus, the proliferation of gastric glands (Walsh et al., 2008). In the human gastrointestinal tract, TGF- β 1 plays the crucial role of mediator in epithelial cell interactions where it regulates epithelial cell proliferation, inflammation and tissue repair. Its expression increases after acute epithelial injury and in patients with inflammatory bowel disease. TGF- β 1 null mice died 20 days after birth because they caught a multifocal inflammatory disease in the stomach and intestine (Frey & Polk, 2006; Hellmich & Evers, 2006). Ethanol ingestion may activate the innate immune response which results in releasing the proinflammatory cytokines as TNF- α and IL-6.

Previous studies associated the level of TNF- α with increased gastric tissue damage induced by ethanol (Li et al., 2013b; Mei et al., 2012). Stimulation of TNF- α has a disadvantageous effect which includes stimulated tissue injury and inflammation. These effects have been reported in a previous study that mentioned the abnormal elevated level of TNF- α that was noticed in the serum of mice with inflammatory diseases including acute mucosal inflammation (Wise & Yao, 2003). This present study demonstrated the pretreatment with *P. macrocarpa* and *T. crista* could remarkably decrease TNF- α in ethanol induced gastric ulcer and thus cause a decrease in the gastric ulcer lesions due to

alleviating gastric mucosal inflammation. This result agrees with previous studies (Costa et al., 2013; Mei et al., 2012). Decreasing levels of TNF- α in pretreated groups are a result of the immune suppressor effect on elevated TGF- β 1 (Kuemmerle et al., 2012).

5.1.4 Wound healing evaluation parameters

Cutaneous wound repair follows an ordered and definable sequence of biological events, beginning with wound closing, developing, repairing and remodeling of damaged tissue (Roy et al., 2009). The management of chronic wounds is a major problem because of the high cost of therapy and presence of undesirable side effects (Umachigi et al., 2007). The main finding of this study is that the topical application of *P. macrocarpa* and *T. crispa* extracts to skin excision wounds in rats resulted in an improved wound contraction rate and a serious reduction in healing time compared to the control group (Figure 4.16 and 4.17). This result may be attributed to the enhanced progression of the wound healing process and the exhibition of noticeable wound margin dehydration as a result of tissue regeneration.

The histological evaluation of wound areas in treated groups confirmed the display of increased cellular infiltration, angiogenesis, increased fibroblasts and collagen deposition (Figure 4.19 and 4.20). The underlying mechanisms of topical *P. macrocarpa* and *T. crispa* action in the wound area which are caused by the chemotactic influence of the plant extract may attract inflammatory cells. The mitogenic activity of the plant extract may increase cellular proliferation and contribute significantly to the healing process. *P. macrocarpa* and *T. crispa* treated groups had significantly smaller wound areas on the 15th day after wounding compared to the vehicle control group. Reactive oxygen species (ROS) are deleterious to the wound healing process because of their harmful effects on cells and tissues. Absorbable synthetic biomaterials are degraded by ROS (Aliyev et al.,

2004). These oxygen species result in damage of the cell. Thus, tissues must be protected from oxidative damage via intracellular and extracellular antioxidants. Free radical scavenging enzymes are a cytoprotective enzymatic group that plays an essential role in the reduction, de-activation and removal of ROS as well as in the regulation of the wound healing process. SOD converts superoxide to hydrogen peroxide which is then transformed into water by the CAT in lysosomes (Johansen et al., 2005). In the current study, homogenate tissue from wounds dressed with *P. macrocarpa* and *T. crispa* showed a significant decrease in the levels of MDA (Figure 4.23) and inflammatory TNF- α (Figure 4.25) indicating a significant elevation in SOD (Figure 4.21) and CAT (Figure 4.22) activity in response to oxidative stress. These findings agree with previous studies (Gouthamchandra et al., 2010; Nevin & Rajamohan, 2010; Singh et al., 2006; Umachigi et al., 2008). The reduced SOD activity in the homogenate wound obtained from the vehicle control group has been attributed to the rise of production in reactive oxygen radicals which could decrease the action of antioxidant enzymes (El-Razek et al., 2012).

The plants have a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids. These metabolites have been screened *in vitro* and display antioxidant, anti-inflammatory and antimicrobial effects which are used to develop drugs or dietary supplements (Ali et al., 2012; Amom et al., 2009; Kongkathip et al., 2002; Yosie et al., 2011). The presence of these antioxidant and anti-inflammatory properties could be one of the factors that contribute to the wound-healing potential of the *P. macrocarpa* and *T. crispa* extracts. The topical application of *P. macrocarpa* and *T. crispa* restored the activities of these antioxidant enzymes which may help to avoid the deleterious effects of free radicals. Several previous studies have reported the utilization of the antioxidant ability of both plants and their ability to induce the activity of

antioxidant enzymes in the prevention or treatment of a variety of diseases such as cancer, diabetes, atherosclerosis, hyperglycemia, and hypercholesterolemia besides having anti-inflammatory ability (Ali et al., 2012; Mohammed et al., 2012; Ruan et al., 2012; Ruan et al., 2013; Susilawati et al., 2012; Winarno, 2012).

TGF- β 1 plays an important role as an inflammatory mediator in the initiation of wound healing by activating and stimulating the macrophage to secrete cytokines that act as fibroblast growth factors, platelet derived growth factors, TNF- α and interleukin1. The proliferative phase TGF- β 1 level was elevated as secreted by the macrophage, T lymphocytes and platelets. TGF- β 1 is believed to be a major control signal regulating fibroblast functions. TGF- β 1 has three effects on extracellular matrix precipitation: it enhances the gene transcription of collagen, fibronectin and proteoglycans, which are important in the production of matrix proteins, inhibits the production of proteases in charge of matrix breakdown and stimulates the metalloprotease inhibitor (Diegelmann & Evans, 2004).

Wounds heal from granulated tissue that serves to fill the dermal defect and provides a structural framework for the deposition of newly synthesized collagen. The substrates and inducers provided for re-epithelialization of the wound may also induce the influx of macrophages and proliferation of mesenchymal cells and capillaries that compose the granulation tissue (Davidson et al., 1985). It has been postulated that by applying agents that induce fibroblast and/or endothelial cell proliferation to heal impaired wounds may increase the rate and degree of granulation tissue formation and stimulate wound repair (Grotendorst et al., 1985). In response to tissue loss, fibroblasts proliferate and migrate into the defect until the wound is populated by fibroblasts and extracellular matrix (Kwon et al., 2007). Cellular contraction is more important early on than collagen contraction in reducing the diameter of the wound (Al-Bayaty & Abdulla, 2012).

Many studies concerned with the use of medicinal plants in treating wound healing have reported the important and crucial role of TGF- β 1 in the accelerated wound healing process. The use of *Eleutherine indica* in the treated wound, accelerates wound healing through the effect of the stimulation of released TGF- β which is bound to the fibroblast receptors and initiates TGF- β -Smad mediated collagen production. Whereas Smad is, family proteins to TGF- β type 1 receptor so that transduction the receptor signals to specific target gene in the nucleus interact directly with activated TGF- β receptors (Upadhyay et al., 2013). Similar results were produced with *Polygonum cuspidatum* in the treated wound. The immunohistochemistry results showed that TGF- β 1 increased significantly in the group treated with plant extract on day 1, 3 and 7 after wounding (Wu et al., 2012). Others studies have revealed that the increased release of TGF- β 1 improved wound healing (Ganeshkumar et al., 2012; Tang et al., 2007; Wang et al., 2012b). This was also observed in our study with a significant elevation in the level of TGF- β 1 in groups treated with *P. macrocarpa* and *T. crispa* extract compared to the vehicle control group and a significant reduction in the level of TNF- α on day 15 after wounding. These results clearly indicate that the effect of both plants in accelerating wound healing may be due to the effect of the rapid movement to the proliferative phase and the shortening of the inflammatory phase to enhance wound contraction. To provide supplementary evidence for this suggestion, previous studies have reported that during the second and third week of healing, fibroblasts start to adopt myofibroblast phenotype properties via large parcels of actin containing microfilaments which are organized along the cytoplasmic cover of the plasma membrane, establishing cell to cell and cell matrix linkages (Hinz et al., 2004; Welch et al., 1990).

In some wound cases, myofibroblasts express smooth muscle actin (Desmouliere et al., 1995). Essentially, TGF- β 1 stimulates cultured human fibroblasts to express smooth

muscle actin and may be responsible for its expression *in vivo* (Gabbiani, 2003). Aspects of the biomechanics of extracellular matrix contraction are the crosslink between actin bundle arrays, cell-to-cell, cell matrix linkages and collagen. The contraction process requires a cytokine signal such as TGF- β 1 or PDGF. As TGF- β 1 was found persistently in the dermal wound during contraction of the tissue it is the most probable candidate for the stimulation of wound contraction (Singer & Clark, 1999). TGF- β 1 plays an important function in the modulation of collagenase and as tissue inhibitor of metalloproteinase expression *in vivo*; they both have an important role during tissue formation and remodeling process (Langholz et al., 1995).

5.1.5 Effect on BAX and BCL2 gene expression

Apoptosis is the active process of eliminating cells. Specific signal molecules and enzymes initiate and regulate this process. Apoptosis is important in therapeutic manipulation and potentially plays a role in reducing inflammation by removing inflammatory cells and assisting to change the fibro proliferative mass in cellular scar tissue in wound healing (Mutsaers et al., 1997). Activation of caspases-8, -9 and -7 in wounded mice on day 3 after injury indicates that the apoptotic pathway may affect cellular elimination during skin wound healing (Zhao et al., 2009). Apoptosis occurs late in the normal wound healing process to form scar tissue and reduce cell proliferation for myofibroblast phenotype and procollagen I mRNA expression. Abnormal control of apoptosis cell death may be the cause of impaired wound healing as seen in the diabetic model (Darby et al., 1997). However, the reduced fibroblast activity from collagen gel is not associated with cell death but many fibroblasts on day 10 of the wound healing process formed pyknotic nuclei which are a cytological marker for apoptosis (Desmouliere et al., 1995). Others have suggested that apoptosis is a mechanism responsible for the transition from fibroblast rich granulation tissue to a relatively cellular scar (Desmouliere et

al., 1997). The apoptosis signals for wound fibroblasts have not been explained. Fibroplasia in wound repair is strongly regulated while in fibrotic diseases such as keloid formation and scleroderma, the fibroplasia process is under a dysregulated state. The latest evidence suggests that fibroblast apoptosis signals in keloids can be obstructed either directly or indirectly (Linge et al., 2005).

In our study, the Fold change ($2^{-\Delta\Delta CT}$) of real time PCR analysis to the mRNA expression level of the BAX and BCL2 on the 15th day after wound, showed up-regulation in the expression of the BAX gene in the reference drug, *P. macrocarpa* treated group at doses of (100mg/ml) and *T. crispa* at both doses (100, 200) mg/ml with fold change (1.0604, 1.1933, 1.4276 and 1.0539) respectively. While the results showed down regulation in the *P. macrocarpa* treated group at a dose of (200 mg/ml) with fold change (1.0141) when compared to the calibrator group, down-regulation happened in the BCL2 genes in the reference drug group, *P. macrocarpa* and *T. crispa* treated groups at both doses (100 and 200) mg/ml with fold change (1.1358, 1.0461, 1.3102, 1.0412 and 1.7144) compared to the calibrator group (Table 4.7). These findings may be an indication that apoptosis signals the end of the inflammatory phase of healing. This data also indicates that the apoptosis of granulation tissue begins at wound closure and decreases when the wound is healed or closed at about 95%. As the group with topical treatment of *P. macrocarpa* at a dose of 200 mg/ml showed the beginnings of down regulation of the BAX gene expression, this may indicate a return to the normal situation of a balance between pro-apoptosis and anti-apoptosis genes. In addition, these findings agree with the gradual granulation tissue absorption observed at wound closure. These findings confirm the results of a previous study by Kane et al., which suggested that an inverse relationship existed between BCL2 and p53. The expression of BCL2 was increased to allow cellular proliferation to occur at the beginning of the injury, whereas p53 expres-

sion decreased. As the inflammation process declined, the p53 level increased as opposed to a decrease in the BCL2 level (Kane & Greenhalgh, 2000). Desmouliere et al. investigated the role of apoptosis in the transformation of granulation tissue to scar tissue in their work and suggested that the peak of apoptosis is between 12 days to 25 days. The generation of apoptotic myofibroblasts and vascular cells were highest during this period (Desmouliere et al., 1995). Multiple apoptosis related factors are involved in skin wound healing, the peak of expression in these factors is between 3 to 7 days after injury, and the expression is in decline, so that the apoptosis process is elevated at the start of the wound healing process and reduced at the end of the wound healing process. The time dependent expressions of the factors in the apoptotic pathway during skin wound healing may be used as potential markers for wound age estimation (Zhao et al., 2009). In the first stage of the wound healing process, fibroblasts produce a preliminarily cell mass and keratinocytes that are responsible for cell production at a certain stage, to lead first to the growth of less undifferentiated cells, which differentiate along the lifetime to become highly complex barrier cells. This process is associated with cell apoptosis in the final stage. Finally wound healing is successfully completed (Houghton et al., 2005). Throughout wound healing, myofibroblasts have a crucial role in matrix formation and wound contraction which fades by apoptosis at the end of healing. In a normal human wound compared to a hypertrophic scar, there was found to be a decrease in levels of anti-apoptosis protein BCL2 while there was an increase in the level of pro-apoptosis protein BAX in normal wound healing. However, in the case of the hypertrophic scar, the level of both protein BCL2 and BAX was reversed from what it was in normal wound healing. These results indicate that disorder in apoptosis can cause the disappearance of myofibroblasts at the end of wound healing that leads to scar formation (Moulin et al., 2004).

Fluctuation of apoptosis in wound healing might be a crucial reason for delayed wound healing. The expression of BAX protein increased similarly with increasing apoptosis and revealed a good compatible relationship with the wound healing process whereas the expression of BCL2 protein declined apoptosis. BAX and BCL2 proteins play an important role in the apoptosis pathway in regulating wound healing (Cui et al., 2003).

5.1.6 Immunomodulatory effect of *P.macropcarpa* and *T. crispa* on the RAW264.7 cell line

Modulation of the immune system refers to any alteration in immune response that can include stimulation, expression or embarrassment of some percentage or stage of the immune response. Any biological or synthetic element used for its influence on the immune system is an immunomodulator. Immunosuppressants and immunostimulators are two common types immunomodulators based on their properties that have the capacity to increase an immune response or defend against pathogens or tumors (Saroj et al., 2012). Substances that have been shown to modify the immune systems are immunomodulators which respond to threats against the system. They modulate and potentiate the weapons of the immune system by keeping them in a highly prepared state for any threat that it may encounter (Mishra et al., 2008). Modulation of the immune response to reduce diseases has long been of interest. There are many recent studies on ethnomedicinal plants as immunomodulatory agents. A relatively new and developing branch of pharmacology is immunopharmacology which aims to search for immunomodulators. The probable use of immunomodulators in clinical medicine comprises the reconstruction of immune deficiency (Alamgir & Uddin, 2010). There has been much research on plant extracts in different parts of the world for their possible immunomodulatory properties. Some of these studies have demonstrated the isolation of potential

bioactive molecules (Alamgir & Uddin, 2010). Shosaiko-to, a Japanese herb, possesses many ethopharmacological effects and these effects are via the modulation of numerous host immune responses. Researchers have suggested that its effect in hepatitis B might be because of its ability to stimulate INFs and activate natural killer cell activity (Borchers et al., 2000).

Polysaccharide has been isolated from *Taxillus chinensis* and *Uncaria rhynchophylla*, two Chinese herbs that have an immunomodulatory effect by stimulating the mouse macrophage to produce TNF- α and nitric oxide in a concentration dependent manner (Zhang et al., 2013a). Another plant is Sairei-to, a Japanese – Chinese herbal medicine that has been used to treat skin diseases. A study done to investigate the immunological changes of skin, kidney, spleen and serum in autoimmuneprone mice treated with Sairei-to revealed that this plant can reduce CD19 and the level of IgG proportion in the serum, CD 4/8 ratio in spleen cells, expression of IFN- γ mRNA and the degree of lymphoproliferation, while causing an increase in the IL-4 production of the spleen cells (Ito et al., 2002). Crude oil seeds of *Nigella sativa* have been used for thousands of years as a spice and food preservative. Many studies have reported its immunomodulatory beneficial properties through increasing T cells and natural killer cell mediated immune responses. *N. sativa* oil showed an increase in 55% CD4/CD8 T cells ratio and a 30% rise in natural killer cell function in humans treated with its oil for four weeks (Salem, 2005). Macrophages are the first line of defense in innate immunity against microbial infection, professional in phagocytes engulf, kill microorganisms and present antigens for exciting adaptive immune responses (Girotti et al., 2004). Macrophages play a main function in tissue remodeling through development, wound healing and tissue homeostasis. In addition, it is essential to innate immunity and pathology of tissue injury and inflammation (Robert et al., 2011). Through phagocytosis, macrophages se-

crete cytokines as interleukins, TNF- α , IFNs and inflammatory mediators like nitric oxide (Macmicking et al., 1997; Stojanovic et al., 2011). Macrophages are central in maintaining homeostasis and have a chief function in host defense against pathogens and in attacking cancer cells (Gamal-Eldeen et al., 2006).

To achieve the *P.macropcarpa* and *T. crispa* and their isolated fractions task in activated macrophage to release immunomodulatory cytokines as IFN- γ , IL-6 and IL-8. a RAW267.4 macrophage cell line was used in our study to determine the immunomodulatory activities by investigating their intracellular cytokine production.

The results of the study showed that there was an immunomodulatory effect of *P.macropcarpa* and *T. crispa* and their effect fraction by increasing the RAW 264.7 macrophage cells proliferation in a dose dependent manner and significant inducing of the intracellular expression of cytokines IFN- γ , IL-6 and IL-8. These findings clearly indicate the significant immunomodulatory effect as immunostimulators of both plants and their active fractions (Figure 4.31, 4.32 and 4.33). These findings are valuable and point to both isolated fractions from *P.macropcarpa* and *T. crispa*, as very appropriate candidates for modulation of macrophage function and inducing the immune system. It is important to note from the literature that immunomodulatory action plays a crucial role in antitumor activity (Abu et al., 2014; He et al., 2012; Zhang et al., 2013a). Therefore, the active isolated fractions from both *P.macropcarpa* and *T. crispa* are potential candidates for antitumor efficacy. Preliminary studies on the antiproliferative efficiency of *P.macropcarpa* and *T. crispa* extracts support this hypothesis (Faried et al., 2007; Ibahim et al., 2011; Radji et al., 2010; Tungpradit et al., 2010). Response to tissue injury is called inflammation, this response is categorized in the acute phase by increasing blood flow and vascular permeability with accumulation of fluid, leukocytes and inflammatory mediators as cytokines. In the chronic phase, the immune response characteristics are

the development of specific humoral and cellular immune responses to pathogens that exist at the site of tissue injury. Many agents participate in leukocyte recruitment by increasing expression of cellular adhesion molecules and chemoattraction during the course of both the acute and chronic inflammation processes. Varieties of these soluble mediators regulate the activation of the resident cells such as fibroblasts, endothelial cells, tissue macrophages and mast cells. In addition, their effect in activating newly staffing inflammatory cells includes monocytes, lymphocytes, neutrophils and eosinophils (Feghali & Wright, 1997). Our results clearly revealed that treatment with both plants and their fractions could enhance the immune response and stimulate the production of essential mediator cytokines such as $\text{INF-}\gamma$, IL-6 and IL-8; these play an important and main role in the acute and chronic inflammatory response. Stimulation of acute phase protein synthesis by the liver is through IL-6, and acts as a growth factor for mature B cells and stimulates their final maturation into antibody producing plasma cells; this involves T cell activation and differentiation and its effect in the induction of IL-2 receptor expression and in addition its role in acute phase response, chronic inflammation, autoimmunity, fibrogenesis and endothelial cell dysfunction (Barnes et al., 2011; Martín-Cordero et al., 2011). $\text{INF-}\gamma$ effects are identified in murine kupffer cells, stimulated macrophages and share in the development of Th1 cells. Besides this, the cellular effects include up-regulation of pathogen recognition, antigen processing and presentation, antiviral case, inhibition of cellular proliferation, effect on apoptosis and immunomodulation (Schroder et al., 2004). IL-8 has potential as a neutrophil chemotactic factor. Many types can produce a large amount of IL-8 in response to a variety of stimuli such as proinflammatory cytokines, microorganisms and their products and environmental alteration including hypoxia and hyperoxia. IL-8 is a main mediator in neutrophil mediated acute inflammation and has a varied range of actions on different types

of cells which include endothelial cells, fibroblasts, monocytes, lymphocytes and neutrophils. These functions suggest that IL-8 has an important function in different pathological disorders such as chronic inflammation and cancer (Mukaida, 2003).

On a similar note, many previous studies have revealed the same effect in medicinal plants, algae or their derivative compounds. The polysaccharide-sulfated xylomannans isolated from *Nemalion helminthoides* can stimulate macrophage cells and promote secretion of cytokines in both RAW 264.7 cells and mice. In addition, it can act as an immunoprotector against a herpetic infection. These results suggest the possibility of using polysaccharide in immunopotentiator supplements or vaccine adjuvants (Pérez-Recalde et al., 2014). *Astragalus membranaceus* polysaccharide could improve immune response in the host individual by inducing the secretion of IL-2, IL-12 and TNF- α besides inhibiting tumor progression *in vivo* (Yang et al., 2013). The same effect of *Sargassum fusiforme* promotes immune response and can inhibit the growth of lung adenocarcinoma in mice (Chen et al., 2012).

Immunosuppression is one of the major problems in chemotherapy and radiotherapy. Thus, it is important to find a new antitumor drug that can enhance immune response. Activated macrophages play a crucial role in the immune system against tumor growth through their ability in pathogenesis, synthesis, and release of nitric oxide and H₂O₂ that are believed to be cytotoxic against particular tumors (Alonso-Castro et al., 2012). Besides improved immune cell function, natural killer cells play an important role in immune surveillance by the secretion of cytokines such as IFN- γ (Brutkiewicz & Sriram, 2002). *Kadsura marmorata* polysaccharide could stimulate Th1 cells to produce IL-2, IFN- γ , and TNF- α . In turn, Th1 response can support protective immunity against intracellular infections such as bacteria, viruses and protozoa against cancer cells (Wang et al., 2013). Another research on *Cervus nippon* polypeptide showed the

ability of this polypeptide to stimulate Th1 cells to release cytokines IL-12, IL-2, TNF- α and IFN- γ while causing a decrease in releasing cytokines IL-10 and IL-4 from Th2 cells. These results reveal the importance of the ability to modulate Th1/Th2 arms of immune system and to use this ability for the treatment of Th2 derived pathological disturbances such as AIDS, viral hepatitis, multiple allergies, cancer and other illnesses. If these two arms of the immune system can be balanced through inducing Th1 cells and decreasing Th2 cells, many of the signs associated with these chronic diseases would be reduced or eliminated (Zha et al., 2013).

From our outcomes and previous studies, we conclude that we can employ medicinal plants and their isolated compounds as immunomodulators to treat chronic inflammation diseases, autoimmune diseases or any other immune disorder disease and as anti-tumor agents.

5.1.7 Phytochemical investigation of active constituents

The LC-MS results identified five compounds in the *P. macrocarpa* F1 including oxoglucine (C₂₀H₁₇NO₅), gallic acid (C₇H₆O₅), harmaline (C₁₃H₁₄N₂O), myricetin (C₁₅H₁₀O₈) and orotic acid (C₅H₄N₂O₄). Previous studies reported isolating many phenolic and flavonoid compounds from fruit and various parts of the plant and their medicinal potential was attributed to these constituents. Flavonoid content from the fruit pericarp of *P. macrocarpa* was analyzed by HPLC and revealed that the fruit has myricetin (Hendra et al., 2011b). Gallic acid isolated from the fruit was investigated and its anticancer effect on a human cancer cell line (Faried et al., 2007). Secondary metabolite studies on *P. macrocarpa* have confirmed the presence of kaempferol-3-O- β -D glucoside in the fruit which protect rats from hepatoma caused by oxidative stress (Zhang et al., 2006). Moreover, Osimi (Oshimi et al., 2006) isolated three compounds (phalerin, mangiferin and icariside C3) from the fruit and showed vasorelaxant activity against

noradrenaline induced contraction of isolated aorta. In addition, benzophenone glucoside was isolated from the fruit and bark (Simanjuntak, 2008; Winarno & Ermin, 2009).

Previous reviews confirmed the medicinal potential of the identified compounds. Oxoglaucine is an alkaloid and has immunomodulation effects. It prevents the increase in CD8 cells that is induced by *Candida albicans* infection in adult mice and inhibits serum accumulation of TNF- α . Thus, treatment of arthritic mice infected with *C. albicans* with oxoglaucine can enhance the host resistance against the pathogen (Ivanovska & Hristova, 2000). Oxoglaucine isolated from *Glaucium flavum* has an antiviral effect against enterovirus (Nikolaeva-Glomb et al., 2010). Other studies have revealed the antiviral properties of oxoglaucine when combined with other enterovirus inhibitors and this leads to a reduction of viral replication and inhibits toxicity (Nikolaeva-Glomb et al., 2011).

Gallic acid is a phenolic acid and is known as an anticancer, proapoptotic, anti-inflammatory and antioxidant agent (Locatelli et al., 2013; Mansouri et al., 2013; Yoon et al., 2013). Harmaline is an alkaloid and has been broadly studied in the last decade and is known to have multiple pharmacological properties involving antimicrobial, antimalarial, antiplasmodial, hypothermic, antitumor, antioxidant and vasorelaxant properties (Ahmad et al., 1992; Berrougui et al., 2006; Di Giorgio et al., 2004; El Gendy et al., 2012).

Myricetin is a natural flavonol which is one of the flavonoid compounds that is known for its powerful antioxidant ability, having a very important role in metabolism, protecting the body from the injury that results from oxidative stress and improving immunity and protection against invading agents (Cao et al., 2013; Faller & Fialho, 2010; Ivanova et al., 2011).

Orotic acid is an organic compound that is very important in the biosynthesis of pyrimidine bases of nucleic acid in living organisms. In the past, it was believed to be part of the vitamin B complex and was called vitamin B₁₃ but now it has been proved not to be a vitamin (Kelley et al., 1970). Besides, it is important in biosynthesis and has received much interest in the area of medicine and drugs. Some metal complexes of orotic acid with platinum, palladium, magnesium and zinc have shown therapeutic properties and are extensively used in medicine as well as potent anticancer agents (Castan et al., 1990; Maistralis et al., 2000; Newman et al., 1989).

The LC-MS results for *T. crispa* F2 identified four compounds that included cordioside (C₂₅H₃₄O₁₁), quercetin (C₁₅H₁₀O₇), eicosenoic acid (pauillinic acid) (C₂₀H₃₈O₂) and boldine (C₁₉H₂₁NO₄). Cordioside was reported to be isolated from *T. crispa* with three other compounds tinosporaside, columbin and β -hydroxyecdysone (Ahmed et al., 2006). Many previous studies have reported the isolation and identification of phenolic compounds, alkaloids, flavonoid, diterpenes and triterpenes from *T. crispa*, and showed their biological activities as highly antioxidant compounds, and their antiproliferative and anti-inflammatory activity (Koay & Koay, 2013; Yusoff et al., 2014). An alkaloid isolated from *T. crispa* had cytotoxic activity against *Toxoplasma gondii* (Lee et al., 2012). Borapetoside C and borapetol B isolated from *T. crispa* showed improved insulin activity (Lokman et al., 2013; Ruan et al., 2012). Praman et al., isolated five compounds (uridine, adenosine, higenamine, salsolinol and tyramine) from *T. crispa* that improved the cardiovascular system and reduce the blood pressure (Praman et al., 2012).

Cordioside and quercetin is a flavonoid compound well known for its powerful antioxidant activity and is employed in many pharmacological applications such as in immunostimulator activities and as an anticancer agent (Govind, 2011; Kapil & Sharma, 1997; Kumar et al., 2011; Lu et al., 2006; Nieman et al., 2009; van Erk et al., 2005).

Eicosenoic acid (paullinic acid) is an omega-7 fatty acid found in a variety of plants. Omega fatty acid is considered an essential fatty acid for human health but unfortunately the body cannot produce it so it must be supplemented with food. Omega fatty acids play an important role in brain function, normal growth and development. Research has proven their effect in reducing inflammation and helping to lower the risk of chronic diseases, which include cancer, heart disease and arthritis. Omega fatty acid deficiency is accompanied by symptoms such as poor memory, fatigue, heart problems, dry skin, poor circulation and depression (Fermor et al., 1992; Harris et al., 2008; Henry et al., 2002; Sen, 2013).

Boldine is an alkaloid; research was conducted during the early 1990s that proved that boldine is one of the most potent natural antioxidants. There are many studies which have focused on its pharmacological properties. The antioxidant properties of boldine have made researchers investigate its effects as anticancer, cytoprotective, anti-inflammatory, anti-atherogenic, anti-diabetic, vasorelaxing and immunomodulator agents (Backhouse et al., 1994; Bannach et al., 1996; Gotteland et al., 1997; Jang et al., 2000; Kubinova et al., 2001; O'Brien et al., 2006).

5.2 Conclusions

This study provides evidence that *P. macrocarpa* and *T. crispa* possess immunomodulatory potential, gastroprotective effect and wound healing activity. Our results indicate that *P. macrocarpa* and *T. crispa* pretreatment has protective effects against ethanol-induced gastric ulcer in rats. Moreover, these results provide evidence that the protective effects of both plants are carried out through the significant effects of some inflammatory mediators, such as PGE₂, gastrin, TGF- β 1, and TNF- α . Moreover, important antioxidant enzymes such as SOD and CAT are scavengers of ROS and can prevent gastric injury. This is associated especially with protection of gastric mucus excretion by enhancing mechanisms to protect the gastric mucosa from offensive factors and by decreasing gastric acidity, increasing production of mucus, decreasing the level of gastrin, pepsin, TNF- α and MDA and increasing the PGE₂ level. In addition, the antioxidant ability has the effect of decreasing cell damage.

The study suggests that *P. macrocarpa* and *T. crispa* have significant excision wound-healing potential. Topical treatment with *P. macrocarpa* and *T. crispa* extracts improved the activity of endogenous antioxidants through the elevated activity of SOD and CAT that prevented free radical-mediated tissue injury. These extracts also play an important function in the inflammation process through increasing the level of TGF- β 1 and decreasing TNF- α . In addition, another important role is the regulation of apoptosis gene expression (BAX and BCL2) and its efficacy in the remodeling phase in wound healing. The effectiveness of *P. macrocarpa* and *T. crispa* in the acceleration of wound healing in rats may be due to antioxidant enzymes and a decrease in the MAD level that indicates a decrease in damage to the cell. Besides, the results clearly indicate the effect of *P. macrocarpa* and *T. crispa* in accelerating the wound healing process and this may

be due to the implementation of the rapid movement to the proliferative phase and shortening the inflammatory phase to enhance wound contraction.

The most important finding of this study is the immunomodulatory potential of *P. macrocarpa* and *T. crispa* through enhanced intracellular expression of IFN- γ , IL-6 and IL-8 cytokine in the RAW264.7 macrophage cells. These results clearly indicate the potent effect of *P. macrocarpa* and *T. crispa* in improving the immune response and their effect in the expression of inflammatory mediators such as PGE₂, gastrin, TGF- β 1 and TNF- α that contributes to the gastroprotective ability and wound healing potential shown by *P. macrocarpa* and *T. crispa*.

The LC-MS phytochemical investigation results for *P. macrocarpa* and *T. crispa* reinforce our results, which show that *P. macrocarpa* and *T. crispa* possess compounds known for their immunomodulatory, cytoprotective and antioxidant properties. In conclusion, the results of the study obviously indicate that *P. macrocarpa* and *T. crispa* are safe and have immunomodulatory, gastroprotective and wound healing potential. Therefore, both plants could be considered candidate drugs that require further development and pharmacological studies to investigate their therapeutic potential.. Moreover, the results are in agreement with the scientific basis of using traditional medicinal plants to discover new drugs.

5.3 Future work

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

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APPENDIX I: TISSUE PROCESSING AND STAIN:

I-PREPARE THE TISSUE FOR PARAFFIN PROCESSED:

A- FIXATION:

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

B- TISSUE PROCESSING (AUTOMATED TISSUE PROCESSING, LEICA TP1020):

1- FIXATION:

10% buffered formalin I for 1 hour

10% buffered formalin II for 1 hour

2-DEHYDRATION:

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

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

Appendices

3- CLEANING

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

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

4- INFILTRATION

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

II-HEMATOXYLIN-EOSIN (H&E) STAINING PROTOCOL

A-MAIN STEPS OF STAINING:

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

Appendices

B- STAINING WITH HAEMATOXYLIN & EOSIN:

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

APPENDIX II: MANUFACTURE INSTRUCTION OF KITS AND PROCEDURES

A- PGE₂ (CAYMAN PGE₂ ASSAY ELISA KIT; CAT# 400144)

5. Prepare SPE (C-18) columns by rinsing with 5 ml methanol followed by 5 ml deionized water. Do not allow the SPE Cartridge (C-18) to dry.
6. Apply the sample to the SPE Cartridge (C-18) and allow the sample to completely enter the packing material.
7. Wash the column with 5 ml deionized water. Discard the wash.
8. Elute the PGE₂ from the column with 5 ml ethyl acetate containing 1% methanol. Higher recovery and better reproducibility may be obtained if the sample is applied and eluted by gravity. The wash steps may be performed under vacuum or pressure.
9. Evaporate the ethyl acetate to dryness under a stream of nitrogen. It is very important that all of the organic solvent be removed as even small quantities will adversely affect the EIA.
10. To resuspend the sample, add 500 µl EIA Buffer. Vortex. It is common for insoluble precipitate to remain in the sample after addition of EIA Buffer; this will not affect the assay. This sample is now ready for use in the EIA.
11. Use 50 µl of the resuspended sample for scintillation counting.

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

PGE₂ Express EIA Standard

Reconstitute the contents of the PGE₂ Express EIA Standard (Item No. 400144) with 1.0 ml of EIA Buffer. The concentration of this solution (the bulk standard) will be 10 ng/ml. Stored at 4°C; this standard will be stable for up to four weeks.

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

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

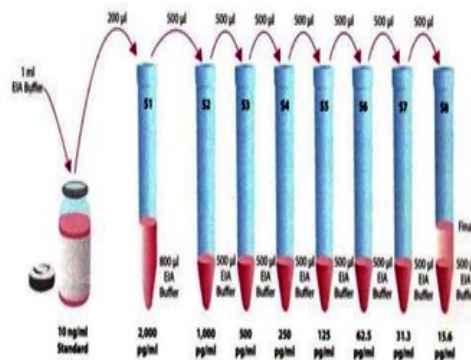


Figure 5. Preparation of the PGE₂ standards

Performing the Assay

Pipetting Hints

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

Addition of the Reagents

1. EIA Buffer

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

2. Prostaglandin E_2 Express EIA Standard

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

3. Samples

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

4. Prostaglandin E_2 Express AChE Tracer

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

5. Prostaglandin E_2 Express Monoclonal Antibody

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

Well	EIA Buffer	Standard/ Sample	Tracer	Antibody
Blk	-	-	-	-
TA	-	-	5 µl (at devl. step)	-
NSB	100 µl	-	50 µl	-
B ₀	50 µl	-	50 µl	50 µl
Std/Sample	-	50 µl	50 µl	50 µl

Table 1. Pipetting summary

Incubation of the Plate

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

Development of the Plate

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

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

OR

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

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

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

Reading the Plate

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

ANALYSIS

Many plate readers come with data reduction software that plot data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as either %B/B₀ versus log concentration using a four-parameter logistic fit or as logit B/B₀ versus log concentration using a linear fit. *NOTE: Cayman has a computer spreadsheet available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/analysis/eia) to obtain a free copy of this convenient data analysis tool.*

Calculations

Preparation of the Data

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

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

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B₀ wells.
3. Subtract the NSB average from the B₀ average. This is the corrected B₀ or corrected maximum binding.
4. Calculate the B/B₀ (Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B₀ (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain %B/B₀ for a logistic four-parameter fit, multiply these values by 100.)

NOTE: The total activity (TA) values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool; the corrected B₀ divided by the actual TA (10X measured absorbance) will give the % Bound. This value should closely approximate the % Bound that can be calculated from the Sample Data (see page 28). Erratic absorbance values and a low (or no) % Bound could indicate the presence of organic solvents in the buffer or other technical problems (see page 32 for Troubleshooting).

B- SOD (CAYMAN, CAT#. 706002)

ASSAY PROTOCOL

Plate Set Up

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

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

A-G = Standards

S1-S41 = Sample Wells

Figure 2. Sample plate format

Pipetting Hints

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

General Information

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

Standard Preparation

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

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

Table 1. Superoxide Dismutase standards

Performing the Assay

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

ANALYSIS

Calculations

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

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

Performance Characteristics

Precision:

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

Assay Range:

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

C- CAT (CAYMAN CAT#. 707002)**Standard Preparation**

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

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

Table 1

*Final formaldehyde concentration in the 170 μ l reaction.

Performing the Assay

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

D- MDA (CAYMAN CAT#. 10009055)

Pipetting Hints

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

General Information

- All reagents except samples must be equilibrated to room temperature before beginning the assay. The SDS Solution will take at least one hour to equilibrate to room temperature if stored at 2-8°C. Briefly heating the SDS Solution at 37°C will re-dissolve the precipitated SDS. The SDS Solution can then be stored at room temperature.
- The final volume of the assay is 150 µl in all wells.
- The assay is performed at room temperature.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples and standards be assayed at least in duplicate.
- It is recommended that the samples and standards be kept at 4°C after preparation to increase sensitivity and reproducibility.
- Monitor the absorbance at 530-540 nm or read fluorescence at an excitation wavelength of 530 nm and an emission wavelength of 550 nm.

Colorimetric Standard Preparation

Dilute 250 μl of the MDA Standard (Item No. 10009202) with 750 μl of water to obtain a stock solution of 125 μM . Take eight clean glass test tubes and label them A-H. Add the amount of 125 μM MDA stock solution and water to each tube as described in Table 1.

Tube	MDA (μl)	Water (μl)	MDA Concentration (μM)
A	0	1,000	0
B	5	995	0.625
C	10	990	1.25
D	20	980	2.5
E	40	960	5
F	80	920	10
G	200	800	25
H	400	600	50

Table 1. MDA colorimetric standards

Performing the Assay

1. Label vial caps with standard number or sample identification number.
2. Add 100 μ l of sample or standard to appropriately labeled 5 ml vial.
3. Add 100 μ l of SDS Solution to vial and swirl to mix.
4. Add 4 ml of the Color Reagent forcefully down side of each vial.
5. Cap vials and place vials in foam or some other holder to keep the tubes upright during boiling.
6. Add vials to vigorously boiling water. Boil vials for one hour.
7. After one hour, immediately remove the vials and place in ice bath to stop reaction. Incubate on ice for 10 minutes.
8. After 10 minutes, centrifuge the vials for 10 minutes at 1,600 x g at 4°C. Vials may appear clear or cloudy. Cloudiness will clear upon warming to room temperature.
9. Vials are stable at room temperature for 30 minutes.
10. Load 150 μ l (in duplicate) from each vial to either the clear plate (colorimetric version) or to the black plate (fluorometric version).
11. Read the absorbance at 530-540 nm or read fluorescence at an excitation wavelength of 530 nm and an emission wavelength of 550 nm.

ANALYSIS

Colorimetric Calculations

1. Calculate the average absorbance of each standard and sample.
2. Subtract the absorbance value of the standard A (0 μM) from itself and all other values (both standards and samples). This is the corrected absorbance.
3. Plot the corrected absorbance values (from step 2 above) of each standard as a function of MDA concentration (see Table 1, on page 13).
4. Calculate the values of MDA for each sample from the standard curve. An example of the MDA standard curve is shown below in Figure 3.

$$\text{MDA } (\mu\text{M}) = \left[\frac{(\text{Corrected absorbance}) - (y\text{-intercept})}{\text{Slope}} \right]$$

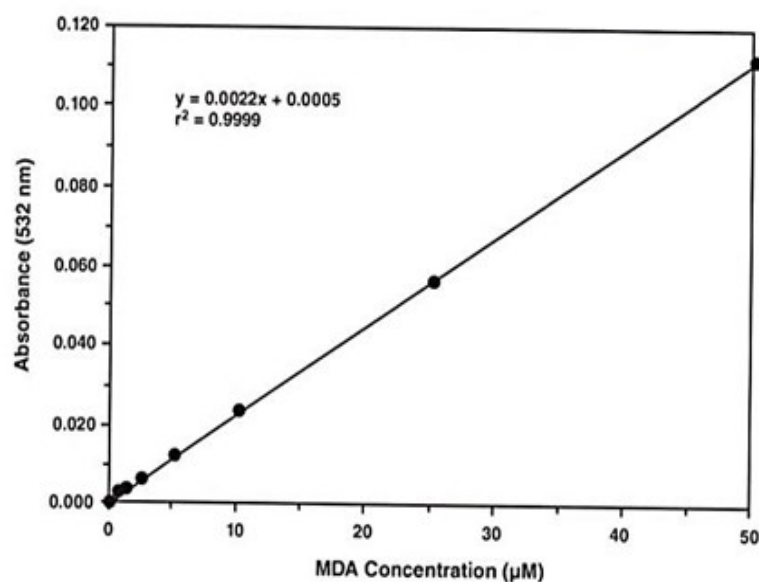


Figure 3. MDA colorimetric standard curve

E- GASTRIN ABNOVA RAT GASTRIN ELISA KIT (CAT#. KA0319 V.01)



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some peptides is improved by the addition of a protease inhibitor cocktail to the sample before freezing.

Extraction of the sample should be carried out using a similar protocol to the one described below.

1. Add an equal volume of 1% trifluoroacetic acid (TFA) in water to the sample. Centrifuge at 17,000 x g for 15 minutes at 4 °C to clarify and save the supernatant.
2. Equilibrate a 200 mg C₁₈ Sep-Pak column with 1 mL of acetonitrile, followed by 10-25 mL of 1% TFA in water.
3. Apply the supernatant to the Sep-Pak column and wash with 10-20 mL of 1% TFA in water. Discard wash.
4. Elute the sample slowly by applying 3 mL of acetonitrile: 1% TFA in water 60:40. Collect the eluant in a plastic tube.
5. Evaporate to dryness using a centrifugal concentrator under vacuum. Store at -20 °C.
6. Reconstitute with Assay Buffer and measure immediately.

Please note that recovery of peptides from extraction processes can be variable. It is important to optimize any process to obtain optimum recoveries. Extraction efficiencies can be determined by a number of methods, including the use of radioactive peptide, or by spiking into paired samples and determining the recovery of this known amount of added Gastrin I.

Note:

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4 °C in the sealed bag provided. The wells should be used in the frame provided.
8. Care must be taken to minimize contamination by endogenous alkaline phosphatase. Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.
9. Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.

Preparation of rat Gastrin I

Allow the 100,000 pg/mL rat Gastrin I standard solution to warm to room temperature. Label seven 12 x 75 mm glass tubes #1 through #7. Pipet 500 µL of standard diluent (Assay Buffer or Tissue Culture Medium) into tube #1. Pipet 250 µL of standard diluent into tubes #2 through #7. Remove 25 µL of diluent from tube #1. Add 25

Rat Gast ELISA kit (Cat # KA0319 V.01)



μL of the 100,000 pg/mL standard to tube #1. Vortex thoroughly. Add 250 μL of tube #1 to tube #2 and vortex thoroughly. Add 250 μL of tube #2 to tube #3 and vortex. Continue this for tubes #4 and #7. The concentration of rat Gastrin I in tubes #1 through #7 will be 5,000, 2,500, 1,250, 625, 313, 156 and 78.1 pg/mL, respectively. See rat Gastrin I Assay Layout Sheet for dilution details. Diluted standards should be used within 60 minutes of preparation.

Gastrin I Conjugate 1:10 Dilution for Total Activity Measurement

Prepare the Conjugate 1:10 Dilution by diluting 50 μL of the supplied conjugate with 450 μL of Assay Buffer. The dilution should be used within 3 hours of preparation. This is intended for use in the Total Activity wells only.

Wash Buffer

Prepare the Wash Buffer by diluting 5 mL of the supplied concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

Protocol

Bring all reagents to room temperature for at least 30 minutes prior to opening. All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.
2. Pipet 50 μL of standard diluent (Assay Buffer or Tissue Culture Medium) into the NSB and the Bo (0 pg/mL Standard) wells.
3. Pipet 50 μL of Standards #1 through #7 into the appropriate wells.
4. Pipet 50 μL of the Samples into the appropriate wells
5. Pipet 25 μL of Assay Buffer into the NSB wells.
6. Pipet 25 μL of blue Conjugate into each well, except the Total Activity (TA) and Blank wells.
7. Pipet 25 μL of yellow Antibody into each well, except the Blank, TA and NSB wells.

NOTE: Every well used should be Green in color except the NSB wells which should be Blue. The Blank and TA wells are empty at this point and have no color.

8. Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired.
9. Empty the contents of the wells and wash by adding 400 μL of wash solution to every well. Repeat the wash 2 more times for a total of 3 Washes.
10. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
11. Add 5 μL of the light blue Conjugate 1:10 dilution (see step 3, Reagent Preparation) to the TA wells.
12. Add 200 μL of the pNpp Substrate solution to every well. Seal plate and incubate at 37 °C for 3 hours.
13. Add 50 μL of Stop Solution to every well. This stops the reaction and the plate should be read



immediately.

14. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

Calculations

Several options are available for the calculation of the concentration of Gastrin I in the samples. We recommend that the data be handled by an immunoassay software package utilizing a weighted 4 parameter logistic curve fitting program. If this type of data reduction software is not readily available, the concentration of Gastrin I can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:
Average Net OD = Average Bound OD - Average NSB OD
2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:
Percent Bound = Net OD x 100 / Net Bo OD x 100
3. Using Logit-Log paper plot Percent Bound (B/Bo) versus Concentration of Gastrin I for the standards. Approximate a straight line through the points. The concentration of Gastrin I in the unknowns can be determined by interpolation.

Typical Results

The results shown below are for illustration only and should not be used to calculate results from another assay.

	Mean	Average	Percent	Gastrin I
Sample	OD (-Blank)	Net OD	Bound	(pg/mL)
Blank OD	(0.104)			
TA	0.352	0.355		
NSB	0.000	-0.003		
Bo	0.276	0.279	100%	0
S1	0.040	0.043	15.6%	5,000
S2	0.053	0.056	20.0%	2,500
S3	0.068	0.071	25.4%	1,250
S4	0.094	0.097	34.8%	625
S5	0.127	0.130	46.6%	313
S6	0.170	0.173	62.0%	156
S7	0.210	0.213	76.4%	78.1
Unknown 1	0.205	0.208	74.8%	84.8
Unknown 2	0.082	0.085	30.3%	842

Rat Gast ELISA kit (Cat # KA0319 V.01)

F- PEPSIN BY CUSABIO RAT PEPSIN ASSAY ELISA KIT (CAT#. CSB-E

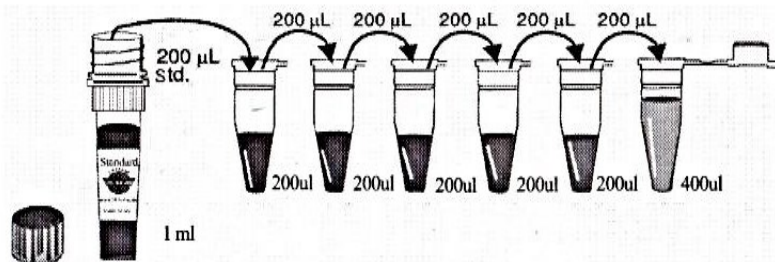
www.cusabio.com

REAGENT PREPARATION

Bring all reagents to room temperature before use for 30min..

1. **Wash Buffer** If crystals have formed in the concentrate, warm up to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to prepare 500 ml of Wash Buffer.
2. **Biotin-antibody** Centrifuge the vial before opening. Dilute to the working concentration using **Biotin-antibody Diluent**(1:100), respectively. The suggested 100-fold dilution can be achieved by adding 10 uL **Biotin-antibody** to 990uL of **Biotin-antibody Diluent** for 1ml working solution.
3. **HRP-avidin** Centrifuge the vial before opening. Dilute to the working concentration using **HRP-avidin Diluent**(1:100), respectively. The suggested 100-fold dilution can be achieved by adding 10 uL **HRP-avidin** to 990uL of **HRP-avidin Diluent** for 1ml working solution.

4. **Standard** Centrifuge the standard vial at 6000-10000rpm for 30s. Reconstitute the **Standard** with 1.0 ml of **Sample Diluent**. This reconstitution produces a stock solution of 200 ng/ml. Allow the standard to sit for a minimum of 15 minutes with gentle and uniform agitation by pipette with 1ml measuring range prior to making serial dilutions. The undiluted standard serves as the high standard (200 ng/ml). The **Sample Diluent** serves as the zero standard (0 ng/ml). Prepare fresh for each assay. Use within 4 hours and discard after use.



Standard	S7	S6	S5	S4	S3	S2	S1
Concentration (ng/ml)	200	100	50	25	12.5	6.25	3.13

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

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- An incubator which can provide stable incubation conditions up to 37°C±0.5°C.

SAMPLE COLLECTION AND STORAGE

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

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

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and controls be assayed in duplicate. All the reagents should be added directly to the liquid level in the well. The pipette should avoid contacting the inner wall of the well.

1. Add 100µl of Standard, Blank, or Sample per well. Cover with the adhesive strip. Incubate for 2 hours at 37°C.
2. Remove the liquid of each well, don't wash.
3. Add 100µl of **Biotin-antibody** working solution to each well. Incubate for 1 hour at 37°C. **Biotin-antibody** working solution may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.
4. Aspirate each well and wash, repeating the process ^{3 wash} three times for a total of three washes. Wash: Fill each well with Wash Buffer (200µl) and let it stand for 2 minutes, then remove the liquid by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel. Complete removal of liquid at each step is essential to good performance.
5. Add 100µl of **HRP-avidin** working solution to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37°C. ^{5 wash}
6. Repeat the aspiration and wash ^{5 wash} five times as step 4.

7. Add 90µl of **TMB Substrate** to each well. Incubate for 10-30 minutes at 37°C. Keeping the plate away from drafts and other temperature fluctuations in the dark.
8. Add 50µl of **Stop Solution** to each well when the first four wells containing the highest concentration of standards develop obvious blue color. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

CALCULATION OF RESULTS

Using the professional soft "Curve Exert 1.3" to make a standard curve is recommended, which can be downloaded from our web.

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the pepsin concentrations versus the log of the O.D. and the

best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

LIMITATIONS OF THE PROCEDURE

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

Appendices

G- TGF-B1 ELISA KIT (ABNOVA, CAT#. KA0279; VERSION 4)



- ✓ Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- ✓ Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.



Assay Protocol

Reagent Preparation

Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.

If crystals have formed in the Buffer Concentrates, warm them gently until they have completely dissolved.

- **Wash Buffer (1x)**

Pour entire contents (50 ml) of the Wash Buffer Concentrate (20x) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water.

Mix gently to avoid foaming.

Transfer to a clean wash bottle and store at 2° to 25 °C. Please note that Wash Buffer (1x) is stable for 30 days.

Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

- **Assay Buffer (1x)**

Pour the entire contents (5 ml) of the Assay Buffer Concentrate (20x) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming.

Store at 2° to 8 °C. Please note that the Assay Buffer (1x) is stable for 30 days.

Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

- **Biotin-Conjugate**

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88



- Streptavidin-HRP

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

- Rat TGF- β 1 Standard

Reconstitute rat TGF- β 1 standard by addition of distilled water

Dilution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 4000 pg/ml).

Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

After usage remaining standard cannot be stored and has to be discarded.

Standard dilutions can be prepared directly on the microwell plate or alternatively in tubes.

External Standard Dilution

Label 7 tubes, one for each standard point.

S1, S2, S3, S4, S5, S6, S7

Then prepare 1:2 serial dilutions for the standard curve as follows:

Pipette 225 μ l of Assay Buffer (1x) into each tube.

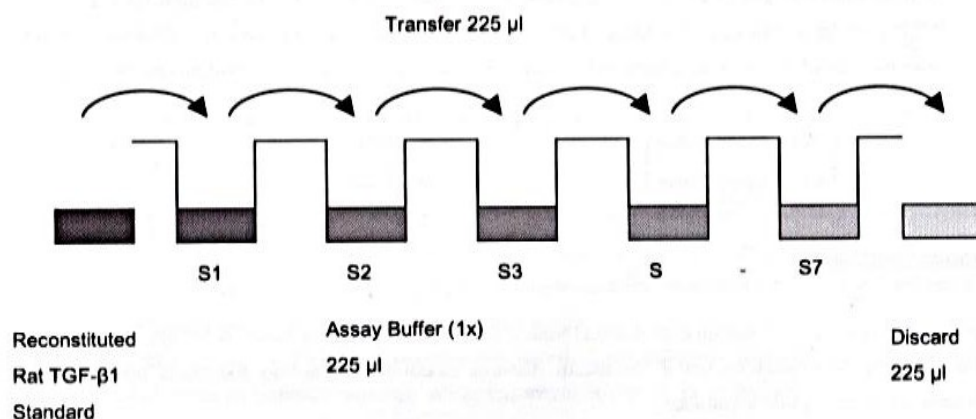
Pipette 225 μ l of reconstituted standard (concentration of standard = 4000 pg/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 2000 pg/ml).

Pipette 225 μ l of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer.

Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 7).

Assay Buffer (1x) serves as blank.

Figure 7



• **Addition of Colour-giving Reagents: Blue-Dye, Green-Dye, Red-Dye**

In order to help our customers to avoid any mistakes in pipetting the Abnova ELISAs, Abnova offers a tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure.

This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.

Alternatively, the dye solutions from the stocks provided (Blue-Dye, Green-Dye, Red-Dye) can be added to the reagents according to the following guidelines:

- ✓ **Diluent:** Before standard and sample dilution add the Blue-Dye at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of Blue-Dye, proceed according to the instruction booklet.

5 ml Assay Buffer (1x)	20 μ l Blue-Dye
12 ml Assay Buffer (1x)	48 μ l Blue-Dye
50 ml Assay Buffer (1x)	200 μ l Blue-Dye

- ✓ **Biotin-Conjugate:** Before dilution of the concentrated Biotin-Conjugate add the Green-Dye at a dilution of 1:100 (see table below) to the Assay Buffer (1x) used for the final conjugate dilution. Proceed after addition of Green-Dye according to the instruction booklet: Preparation of Biotin-Conjugate.

3 ml Assay Buffer (1x)	30 μ l Green-Dye
6 ml Assay Buffer (1x)	60 μ l Green-Dye
12 ml Assay Buffer (1x)	120 μ l Green-Dye



- ✓ Streptavidin-HRP: Before dilution of the concentrated Streptavidin-HRP, add the Red-Dye at a dilution of 1:250 (see table below) to the Assay Buffer (1x) used for the final Streptavidin-HRP dilution. Proceed after addition of Red-Dye according to the instruction booklet: Preparation of Streptavidin-HRP.

6 ml Assay Buffer (1x)	24 μ l Red-Dye
12 ml Assay Buffer (1x)	48 μ l Red-Dye

Sample Preparation

Cell culture supernatant *, serum and plasma (heparin and EDTA) were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive rat TGF- β 1. If samples are to be run within 24 hours, they may be stored at 2° to 8°C .

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

* Pay attention to a possibly elevated blank signal in cell culture supernatant samples containing serum components (e.g. FCS), due to latent TGF- β levels in animal serum.

Assay Procedure

1. Prepare your serum and plasma samples before starting the test procedure. Dilute serum and plasma samples with Assay Buffer (1x) according to the following scheme:

20 μ l sample + 920 μ l Assay Buffer (1x)

Add 30 μ l 1N HCl to 940 μ l prediluted sample, mix and incubate for 1 hour at room temperature.

Neutralize by addition of 30 μ l 1N NaOH.

Prepare your cell culture supernatant samples before starting the test procedure. Dilute cell culture supernatant samples with Assay Buffer (1x) according to the following scheme:

20 μ l sample + 180 μ l Assay Buffer (1x)

Add 20 μ l 1N HCl to 200 μ l prediluted sample, mix and incubate for 1 hour at room temperature.

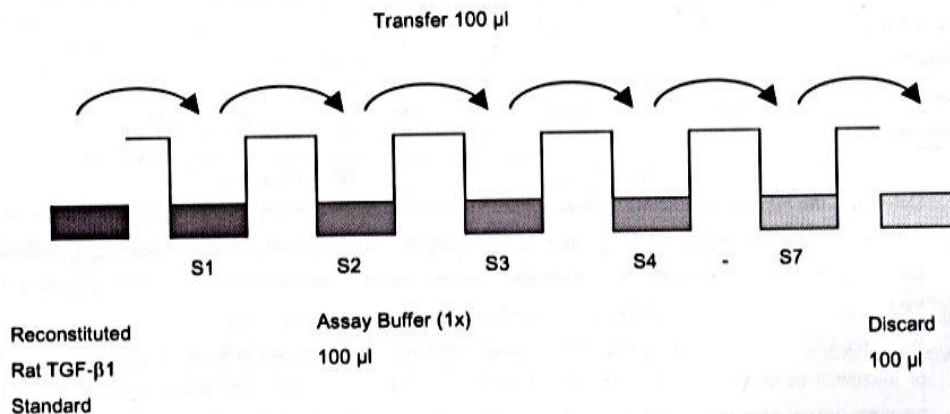
Neutralize by addition of 20 μ l 1N NaOH.



Sample Matrix	Sample Volume (μl)	Assay Buffer (1x) (μl)	HCl 1N (μl)	NaOH 1N (μl)	Dilution
Serum and Plasma	20	920	30	30	1:50
Cell culture supernatant	20	180	20	20	1:12

- Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
- Wash the microwell strips twice with approximately 400 μl Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10 – 15 seconds before aspiration. Take care not to scratch the surface of the microwells.
After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.
- Standard dilution on the microwell plate (Alternatively the standard dilution can be prepared in tubes):
Add 100 μl of Assay Buffer (1x) in duplicate to all standard wells. Pipette 100 μl of diluted standard (concentration = 4000.0 pg/ml) in duplicate into well A1 and A2 (see Plate Layout). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 2000.0 pg/ml), and transfer 100 μl to wells B1 and B2, respectively (see Figure 8). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of rat TGF-β1 standard dilutions ranging from 2000.0 to 31.3 pg/ml. Discard 100 μl of the contents from the last microwells (G1, G2) used.

Figure 8



In case of an external standard dilution, pipette 100 μ l of these standard dilutions (S1 – S7) in the standard wells according to Plate layout.

5. Add 100 μ l of Assay Buffer (1x) in duplicate to the blank wells.
6. For serum and plasma samples add 80 μ l of Assay Buffer (1x) to the sample wells.
For cell culture supernatant samples add 60 μ l of Assay Buffer (1x) to the sample wells.
7. For serum and plasma samples add 20 μ l of each pretreated sample in duplicate to the sample wells.
For cell culture supernatant samples add 40 μ l of each pretreated sample in duplicate to the sample wells.
8. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, on a microplate shaker set at 100 rpm. (Shaking is absolutely necessary for an optimal test performance.)
9. Prepare Biotin-Conjugate.
10. Remove adhesive film and empty wells. Wash microwell strips 5 times according to point 3. of the test protocol. Proceed immediately to the next step.
11. Add 100 μ l of Biotin-Conjugate to all wells.
12. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 1 hour, on a microplate shaker set at 100 rpm. (Shaking is absolutely necessary for an optimal test performance.)
13. Prepare Streptavidin-HRP.
14. Remove adhesive film and empty wells. Wash microwell strips 5 times according to point 3. of the test protocol. Proceed immediately to the next step.
15. Add 100 μ l of diluted Streptavidin-HRP to all wells, including the blank wells.
16. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour, on a microplate shaker set at 100 rpm. (Shaking is absolutely necessary for an optimal test performance.)
17. Remove adhesive film and empty wells. Wash microwell strips 5 times according to point 3. of the test protocol. Proceed immediately to the next step.

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18. Pipette 100 µl of TMB Substrate Solution to all wells.
19. Incubate the microwell strips at room temperature (18° to 25°C) for about 30 min. Avoid direct exposure to intense light.

The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay.

It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

20. Stop the enzyme reaction by quickly pipetting 100 µl of Stop Solution into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
21. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.



Data Analysis

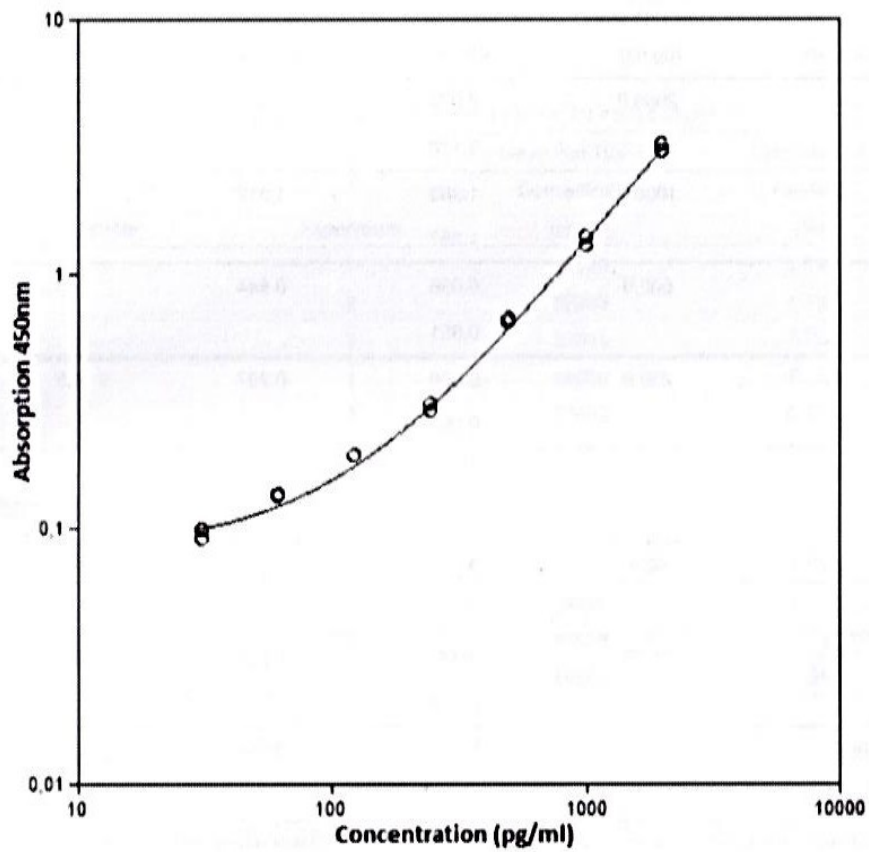
Calculation of Results

- ✓ Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean value.
- ✓ Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the rat TGF- β 1 concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- ✓ To determine the concentration of circulating rat TGF- β 1 for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding rat TGF- β 1 concentration.
- ✓ If instructions in this protocol have been followed serum and plasma samples have been diluted 1:250 (20 μ l sample + 920 μ l Assay Buffer (1x) (= 1:50) + 30 μ l 1N HCl + 30 μ l 1N NaOH and 20 μ l pretreated sample + 80 μ l Assay Buffer (1x) (= 1:5)) and cell culture supernatant samples have been diluted 1:30 (20 μ l sample + 180 μ l Assay Buffer (1x) + 20 μ l 1N HCl + 20 μ l 1N NaOH (= 1:12) and 40 μ l pretreated sample + 60 μ l Assay Buffer (1x) (=1:2.5)), the concentration read from the standard curve must be multiplied by the dilution factor (x 250 or x 30, respectively).
- ✓ Calculation of samples with a concentration exceeding standard 1 way result in incorrect, low rat TGF- β 1 levels. Such samples require further external predilution according to expected rat TGF- β 1 values with Assay Buffer (1x) in order to precisely quantitate the actual rat TGF- β 1 level.
- ✓ It is suggested that each testing facility establishes a control sample of known rat TGF- β 1 concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- ✓ A representative standard curve is shown in Figure 9. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.



Figure 9

Representative standard curve for rat TGF- β 1 ELISA. Rat TGF- β 1 was diluted in serial 2-fold steps in Assay Buffer (1x). Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.



H-TNF- α ELISA KIT (THERMO SCIENTIFIC, CAT#. ER3TNFA)



Sample Preparation

Sample Handling

- Serum, EDTA plasma and culture supernatants may be tested in this assay.
- For each well, 50 μ l of EDTA plasma, culture supernatant or 1:1 diluted serum is required.
- Store samples to be assayed within 2 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -70°C.
- Avoid repeated freeze-thaw cycles when storing samples.
- Test samples and standards must be assayed in duplicate each time the assay is performed.
- Gradually equilibrate samples to room temperature before beginning assay. Do not use a heated water bath to thaw or warm samples.
- Mix samples by gently inverting tubes.
- If samples are clotted, grossly hemolyzed, lipemic or microbially contaminated, or if there is any question about the integrity of a sample, make a note on the template and interpret results with caution.

Sample Dilution

- **Serum and EDTA plasma samples must first be diluted 1:1 before testing.** To prepare a 1:1 dilution, add 100 μ l of sample to 100 μ l of Standard Diluent in a separate tube and mix well. Alternatively, after adding the Pre-Treatment Buffer to the wells add 25 μ l of Standard Diluent to the appropriate sample wells and then add 25 μ l of sample. Tap the plate gently to mix. Either method produces a final 1:1 dilution of the sample in each well.
- If the rat TNF α concentration possibly exceeds the highest point of the standard curve (i.e., 2,500 pg/ml), prepare one or more five-fold dilutions of the test sample. When testing **culture supernatants**, prepare the serial dilutions using culture medium (see Precautions section). When testing **serum or plasma**, prepare the serial dilution using the Standard Diluent provided. For example, prepare a five-fold dilution by adding 50 μ l of test sample to 200 μ l of appropriate diluent. Mix thoroughly between dilutions before assaying.

Reagent Preparation

For procedural differences when using partial plates, look for **(PP)** throughout this instruction booklet.

Wash Buffer

- **(PP)** When using partial plates, store the Wash Buffer at 2-8°C.
 - **Note:** Wash Buffer must be at room temperature before use in the assay. Do not use Wash Buffer that has become visibly contaminated during storage.
1. Label a clean glass or plastic two-liter container "Wash Buffer." The 30X Wash Buffer may have a cloudy appearance.
 2. Add the entire contents of the 30X Wash Buffer (50 ml) bottle to the two-liter container and dilute to a final volume of 1.5 liters with ultrapure water. Mix thoroughly.
- Note:** If using the 5-plate kit, add 30 ml Wash Buffer to 870 ml water for each plate being used.

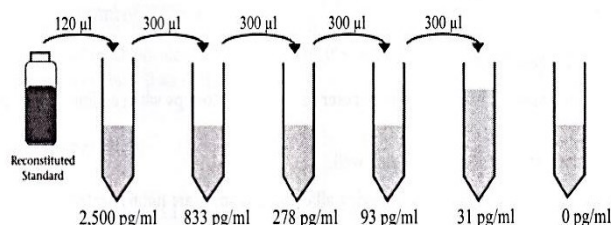
Standards

- **(PP)** Reconstitute and use one vial of the lyophilized Standard per partial plate.
- Note:** When using the 5-plate kit, only one Standard per plate is supplied. Therefore, partial plates cannot be used.
- Prepare Standards just before use and use within one hour of reconstitution. Do not store reconstituted standards.
1. For **culture supernatant** samples, reconstitute standard with ultrapure water to the volume indicated on the standard vial label. The standard will dissolve in approximately 1 minute. Mix by gently inverting vial. Use the sample culture medium to prepare Standard Curve dilutions (see Precautions section).
- For **serum or plasma** samples, reconstitute standard with ultrapure water to the volume indicated on the standard vial label. The standard will dissolve in approximately 1 minute. Mix by gently inverting vial. Use the Standard Diluent provided to prepare standard curve serial dilutions.

When testing **serum or plasma** and **cell culture supernatant** samples on the same plate, validate the media to establish if the same standard curve can be used for both sample types. Prepare a standard curve (including a zero) using culture medium to dilute the standard. Use medium containing serum or other protein to maximize stability of the rat TNF α . Perform this curve in parallel with a standard curve prepared with Standard Diluent. If the OD values of the two curves are within 10% of the mean for both curves, then perform the assay with Standard Diluent, whether testing culture supernatant, plasma or serum samples.

2. Label six tubes, one for each standard curve point: 2,500 pg/ml, 833 pg/ml, 278 pg/ml, 93 pg/ml, 31 pg/ml, and 0 pg/ml. Prepare an initial 1:6 dilution followed by 1:3 serial dilutions for the standard curve as follows:
3. Pipette 600 μ l of appropriate diluent into each tube.
4. Pipette 120 μ l of the reconstituted standard into the first tube (i.e., 2,500 pg/ml) and mix.
5. Pipette 300 μ l of this dilution into the second tube (i.e., 833 pg/ml) and mix.
6. Repeat serial dilutions (using 300 μ l) three more times to complete the standard curve points.

Standard Dilutions Schematic



Assay Procedure

A. Sample Incubation

- (PP) Determine the number of strips required. Leave these strips in the plate frame. Tightly seal the remaining unused strips in the foil pouch with the desiccant provided and store at 2-8°C. After completing the assay, retain the plate frame for the second partial plate. When using the second partial plate, place the reserved strips securely in the plate frame.
- Use the Data Template provided to record locations of the zero standard, rat TNF α standards and test samples. Perform five standard points and one blank in duplicate with each series of unknown samples.

1. Add 50 μ l of the Pre-Treatment buffer to each well.
2. Add 50 μ l of the reconstituted standard or diluted sample to each well in duplicate. Mix by gently tapping the plate several times.

Note: All serum and EDTA plasma samples must be diluted 1:1 before testing (see the Sample Dilution section). If the TNF α concentration in any test sample is expected to exceed the highest point on the standard curve (i.e., 2,500 pg/ml) refer to the Sample Dilution section.

3. Add 50 μ l of Standard Diluent to all wells that do not contain standards or samples.
4. Carefully cover plate with an adhesive plate cover. Ensure that all edges and strips are sealed tightly by running your thumb over the edges and down each strip. Incubate for one (1) hour at room temperature (i.e., 20-25°C).
5. Carefully remove the adhesive plate cover. Wash plate THREE times with Wash Buffer using the procedure described in the Plate Washing section (Section B).

B. Plate Washing

Note: Automated plate washers may produce sub-optimal results. For best results, perform a manual wash the first time the assay is performed. Automated plate washing may require validation to determine the number of washes necessary to achieve optimal results; typically, 3-5 washes are sufficient.

1. Gently squeeze the long sides of plate frame before washing to ensure all strips securely remain in the frame.

2. Empty plate contents. Use a squirt wash bottle to vigorously fill each well completely with Wash Buffer, then empty plate contents. Repeat procedure two additional times for a total of THREE washes. Blot plate onto paper towels or other absorbent material.

Note: For automated washing, aspirate all wells and wash with Wash Buffer, overfilling wells with Wash Buffer. Blot plate onto paper towels or other absorbent material. The number of washes necessary may require optimization.

C. Biotinylated Antibody Reagent Incubation

- If using a multichannel pipettor, **use new reagent reservoir and pipette tips** when adding the Biotinylated Antibody Reagent.
1. Add 50 μ l of Biotinylated Antibody Reagent to each well.
 2. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate plate for 1 hour at room temperature.
 3. Carefully remove the adhesive plate cover, discard plate contents and wash THREE times as described in the Plate Washing section.

D. Streptavidin-HRP Reagent Incubation

- If using a multichannel pipettor, **use new reagent reservoir and pipette tips** when adding the Streptavidin-HRP Reagent.
1. Add 100 μ l of Streptavidin-HRP Reagent to each well.
 2. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate plate for 30 minutes at room temperature.
 3. Carefully remove the adhesive plate cover, discard plate contents and wash THREE times as described in the Plate Washing section (section B).

E. Substrate Incubation and Stop Step

- Use new disposable reagent reservoirs when adding TMB Substrate Solution and Stop Solution.
 - Dispense from bottle **ONLY** the amount required, 100 μ l per well, for the number of wells being used. Do not use a glass pipette to measure the TMB Substrate Solution.
 - **(PP)** Do not combine leftover substrate with that reserved for the second partial plate. Take care not to contaminate remaining TMB Substrate Solution.
 - If using a multichannel pipettor, **use new reagent reservoir and pipette tips** when adding the TMB Substrate Solution and when adding the Stop Solution.
1. Pipette 100 μ l of TMB Substrate Solution into each well.
 2. Allow the color reaction to develop at room temperature in the dark for 10 minutes. Do not cover plate with a plate sealer. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.
 3. After 10 minutes, stop the reaction by adding 100 μ l of Stop Solution to each well.

F. Absorbance Measurement

Note: Evaluate the plate within 30 minutes of stopping the reaction.

Measure absorbance on an ELISA plate reader set at 450 nm and 550 nm. Subtract 550 nm values from 450 nm values to correct for optical imperfections in the microplate. If an absorbance at 550 nm is not available, measure the absorbance at 450 nm only. When the 550 nm measurement is omitted, absorbance values will be higher.

APPENDIX III: RNA CONCENTRATION, PURITY AND PREPARE

2% AGAROSE GEL ELECTROPHORESIS:

A- RNA CONCENTRATION AND PURITY:

RNA concentration and purity ($A_{260}/_{280} \geq 1.65$) measured by Nano Drop ND-2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Sample	RNA ng/ μ l	A260	A280	260/280
Vehicle control 1	396.2	9.906	5.807	1.71
Vehicle control 2	504.1	12.602	6.011	2.1
Vehicle control 3	366.5	9.161	4.321	2.12
Vehicle control 4	370.8	9.271	4.484	2.07
Vehicle control 5	245.1	6.128	2.932	2.09
Reference drug 1	874.6	21.866	10.196	2.14
Reference drug 2	453.2	11.329	5.474	2.07
Reference drug 3	332.5	8.313	3.912	2.13
Reference drug 4	396.9	9.922	4.873	2.04
Reference drug 5	675.1	16.877	8.032	2.1
P.m (100 mg/ml)Sample 1	373.2	9.329	4.588	2.03
P.m (100 mg/ml)Sample 2	531.9	13.297	6.417	2.07
P.m (100 mg/ml)Sample 3	366.7	9.167	4.395	2.09
P.m (100 mg/ml)Sample 4	642.8	16.07	7.683	2.09
P.m (100 mg/ml)Sample 5	701.3	17.533	8.459	2.07
P.m (200 mg/ml)Sample 1	477.8	11.946	5.769	2.07
P.m (200 mg/ml)Sample 2	269.7	6.742	3.26	2.07
P.m (200 mg/ml)Sample 3	235.6	5.89	2.802	2.1
P.m (200 mg/ml)Sample 4	270.4	6.759	3.287	2.06
P.m (200 mg/ml)Sample 5	259	6.474	3.158	2.05
T. c (100 mg/ml)Sample 1	474	11.851	5.715	2.07
T. c (100 mg/ml)Sample 3	315.3	7.883	3.743	2.11
T. c (100 mg/ml)Sample 4	274.9	6.874	3.266	2.1
T. c (100 mg/ml)Sample 5	359.2	8.98	4.291	2.09
T. c (200 mg/ml)Sample 1	282	7.05	3.365	2.1
T. c (200 mg/ml)Sample 2	585.3	14.632	7.025	2.08
T. c (200 mg/ml)Sample 3	514.3	12.857	6.256	2.06
T. c (200 mg/ml)Sample 4	551.1	13.778	6.614	2.08
T. c (200 mg/ml)Sample 5	266.3	6.657	3.152	2.11

Appendices

B- PREPARE 2% AGAROSE GEL ELECTROPHORESIS:

Dissolve (2gm) of agarose in 100ml of 1x Tris-acetate EDTA buffer, then add 1 μ l of ethidium bromide to the mixture and pour on the gel boat to the electrophoresis system. After gel is solidified, load RNA and run the electrophoresis at 95 volts to migrate the RNA bands 28S and 18S.

APPENDIX IV: LIST OF CHEMICALS, CONSUMABLES AND KITS

Chemicals, consumables and kits	Brand	Used in
95% ethanol	Fisher Scientific, UK	Plant extraction
Sodium acetate trihydrate	Sigma-aldrich , Germany	Ferric reducing antioxi- dant power (FRAP)
Glacial acetic acid	Merck, Germany	
HCl	Fisher Scientific, UK	
2, 4, 6-tripyridyl-s-triazine (TPTZ)	Sigma-aldrich, Germany	
FeCl ₃	Sigma-aldrich, Germany	
FeSO ₄ ·7H ₂ O	Sigma-aldrich, Germany	
DPPH (2, 2-diphenyl-1-picrylhydrazyl)	Sigma-aldrich, Germany	Redical scavenging activi- ty test (DPPH)
L-ascorbic acid	Sigma-aldrich, Germany	
Folin-Ciocalteu reagent (phosphomolybdic and phosphotungstic acid)	Merck, Germany	Total phenolic content
Sodium carbonate	Anala R, Engalish	
Gallic acid	Sigma-aldrich, Germany	
Methanol	Fisher Scientific, UK	Estimation of total flavo- noid content
Aluminum chloride	Sigma-aldrich, Germany	
Potassium acetate	Ajax Finechem, Australia	
Quercetin	Sigma-aldrich, Germany	
Tween 20	Sigma-aldrich, Germany	*Acute toxicity test *Gastroprotective ability of the plant extract *Determination of gastric wall mucus content
Xylazine	Ilium, Australia	Histological evaluation of gastric lesions
Ketamine	Ilium, Australia	
Omeprazole	TROGE Medical GMBH, Germany	
Alcian blue	ACROS, USA	
D(+) sucrose	Fisher Scientific, UK	
Sodium acetate	Sigma-Aldrich, Germany	
Magnesium chloride	Sigma-Aldrich, Germany	
Ethyl ether	Anala R, England	
Formalin	Scharlau chemie S.A, Spain	
Paraffin wax	Sigma-Aldrich, Germany	
Hematoxylin stain	Clin-Tech, UK	
Eosin stain	Clin-Tech, UK	

Appendices

Chemicals, consumables and kits	Brand	Used in
PGE2 ELISA kit	Cayman Cat# 400144	Assessment of PGE2
CAT ELISA kit	Cayman Cat#.707002	Assessment of catalase
SOD ELISA kit	Cayman Cat#.706002	Assessment of superoxide dismutase
MDA	Cayman Cat#.10009055	Assessment of malondialdehyde
Bovine serum albumin	JR Scientific, USA	Performance of Protein Concentration by the Bradford method
Bradford reagent	Sigma-Aldrich, Germany	
PBS buffer	DulbeccoA, Oxoid, England	Tissue homogenate sample preparations
Rat gastrin ELISA kit	Abnova Cat#.KA0319 V.01	Assessment of rat gastrin
Rat pepsin assay ELISA kit	Cusabio Cat#.CSB-E 08920r	Assessment of rat pepsin
TGF- β 1 by ELISA kit	Abnova Cat#. KA0279;V.04	Assessment of TGF- β 1
Rat TNF- α by ELISA kit	Thermo Scientific Cat#. ER3TNFA	Assessment of TNF- α
Gum acacia	Sigma-Aldrich, Germany	Wound healing ability of plant extract
Intrasite gel	Trademark of Smith and Nephew Ltd	
RNA solution	Ambion, Austin, Texas, USA	-BAX and BCL2 gene expression in vivo (wound healing model) -RNA extraction
RNA purification kit	Thermo Scientific Cat# K0731	
DNaseI	Qiagen, Hilden, Germany, Cat#:79254	Eliminate genomic DNA contamination
Denaturing agarose	Bio-Rad, Richmond, CA, USA	The RNA concentration and integrity
Ethidium bromide stain	Bio-Rad, Richmond, CA, USA	
High Capacity RNA to cDNA kit	Applied Biosystems, Cat#.4387406	Reverse transcription of total RNA extracted
TaqMan fast advanced master mix	Applied Biosystems Cat#: 4444557	Real time amplification assay
Bcl2-associated X protein (Bax)	Applied Biosystems Rn02532082_g1	
B cell lymphoma -2 (BCL2)	Applied Biosystems Rn99999125_m1	
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous control	Applied Biosystems Rn0177563_g1	
Hypoxanthine phosphoribosyl transferase 1 (HPRT1) endogenous control	Applied Biosystems Rn01527840_m1	

Appendices

Chemicals, consumables and kits	Brand	Used in
silica gel G60, 70 - 230 mesh	Merck, Darmstadt, Germany	Column chromatography
Solvents :hexane, ethyl acetate, methanol, acetonitrile	Fisher Scientific, UK	
A murine macrophage cell line RAW264.7	American Type Culture Collection (ATCC, Rockville, MD)	<i>In vitro</i> stimulation for intracellular cytokine production
DMEM supplemented with 4500g glucose/L, 110mg sodium pyruvate/L and 1% penicillin–streptomycin	Sigma-Aldrich, UK	
fetal bovine serum (FBS)	Biowest, Inc, France	
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay	Merck, Germany	
dimethyl sulfoxide (DMSO)	Fisher Scientific, UK	
LPS (<i>Escherichia coli</i> 055:B5)	Difco, Detroit, MI,USA	
Fixation/ permeabilization solution (BD GolgiPlug™)	BD Cytofix/Cytoperm™ Plus Fixation / Permeabilization, BD Golgi Plug™ protein transport inhibitor, Cat. No.555028	
Sodium azide	Ajax Finerhem, Austerlala	Flow cytometry immunostaining of intracellular cytokines
Mouse BD Fc Block™ purified anti-mouse CD16/CD32 mAb 2.4G2	BD Fc Block™;Cat.No. 553141	
fluorochrome-conjugated anti-cytokine antibody: PE Anti-Mouse IL-6	BD Cat. No: 554401	
APC Rat Anti-Mouse IL-8	BD Cat. No: FAB2164A	
FITC Rat Anti-Mouse IFN-γ	BD Cat. No: 554411	

Appendices

APPENDIX V: LIST OF IDENTIFICATION OF ACTIVE COM- POUNDS FOR *P. macrocarpa* & *T.crispa*

A- Identification of active compounds in *P. macrocarpa*

Number	Compound
1	Benzophenone glucoside
2	DLBS1425
3	Gallic acid
4	Harmaline
5	Icariside C3
6	Kaempferol
7	Mangiferin
8	Myricetin
9	Naringin
10	Orotic acid
11	Oxoglaucine
12	Phalerin
13	Quercetin
14	Rutin

Appendices

B- Identification of active compounds in *T. crista*

Number	Compound
1	Adenosine
2	Boldine
3	Borapetosides A, B and C
4	β -hydroxyecdysone
5	Catechin
6	Columbin
7	Cordioside
8	Cycloeucalenol
9	Cycloeucalenone
10	Epinephrine
11	Higenamine
12	Hordenine
13	Luteolin
14	Morin
15	Paullinic acid
16	Pseudoephedrine
17	Quercetin
18	Rutin
19	Salsolinol
20	Tinosporaside
21	Tyramine
22	Uridine

APPENDIX V: LIST OF PUBLICATION AND PAPERS PRESENTED

A- CONFERENCES

- * Presented Paper at the 17th National Conference on Medical and Health Sciences - Malaysia, May 2012
- * Presented Paper at the International Conference on Medical and Health sciences (IC-MHS), Malaysia, 2013
- * Presented Paper at International Conference on Innovation Challenges in Multidisciplinary Research & Practice on December 2013, Kuala Lumpur, Malaysia

B- PUBLICATIONS AND SUBMITTED PAPERS

- ❖ Walaa Najm Abood, Mahmood Ameen Abdulla, Salmah Ismail. (2014). Involvement of Inflammatory Mediators in the Gastroprotective Action of *Phaleria Macrocarpa* against Ethanol Induced Gastric Ulcer. World Applied Science Journal, 2014: 344-350. DOI: 10.5829/idosi.wasj.2014.30.icmrp.48
- ❖ Walaa Najm Abood, Iman Fahmi Mustafa, Mahmood Ameen Abdulla, Salmah Ismail. (2014). Immunomodulatory effect of an isolated fraction from *Tinospora crispa* on inteacellular expression of IFN- γ , IL-6 and IL-8. BMC Complementary and Alternative Medicine, 2014, 14:205

Submitted papers:

- ❖ Walaa Najm Abood, Mahmood Ameen Abdulla, Salmah Ismail. Evaluation of the Wound Healing Potential of *Tinospora crispa* in Rats: Role of Inflammatory Mediators and Antioxidant Activity, submit to the PLOS ONE Journal.
- ❖ Walaa Najm Abood, Mahmood Ameen Abdulla, Salmah Ismail. Phytochemical Analysis and Immunomodulatory Effect of Isolated a New Bioactive Components from *Phaleria macrocarpa* (Scheff) Boerl) , submit to the Records of Natural Products Journal.