UNIVERSITI PUTRA MALAYSIA

IN-VIVO ANTI INFLAMMATORY STUDIES OF POLYPEPTIDE-K AND ESSENTIAL OIL FROM MOMORDICA CHARANTIA SEEDS (BITTER GOURD)

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ABSTRACT

Inflammation is a physiological response of a body to stimuli including infections and tissue injury. There have been reports that prolonged use of anti-inflammatory drugs (NSAIDs) will lead to some adverse effect. *Momordica charantia* is one of the natural sources that claim having anti-inflammatory properties. This study is to evaluate the effects of polypeptide-k compound and essential oils from *Momordica charantia* seeds on induced inflammation in experimental rats. Anti inflammatory activity of polypeptide-k and essential oils were evaluated by carrageenan-induced paw edema and carrageenan-air pouch model. A total of six groups containing six rats in a group were used throughout the experiment for both models. Group 1 received 5ml/kg distilled water (negative control), group 2 received 10mg/kg indomethacin as positive control meanwhile group 3 and 4 received 10mg/kg and 50mg/kg polypeptide-k compound respectively whereas, group 5 and 6 received 1ml per rat and 2ml per rat of essential oil respectively. All rats in each group were given treatment orally for duration of four days. In the paw edema study, the final oral administration of each treatment was given on the fourth day 30 minutes before 0.1 ml of 1% carrageenan was injected into the hind paw. The paw volume was measured continuously every hour for duration of 5 hours by using plethysmometer. For the air pouch model, the rats were injected subcutaneously with sterile air on their back to form pouches. On day 4, the rats received pretreatment according to their group. 0.5% carrageenan was injected into the pouches to induce inflammation. After 6 hours, the pouches were washed and fluid collected to determine exudates volume, interleukin 10, total and absolute leukocyte counts and nitrite levels. In paw edema study, there is significantly reduction in paw edema volume at 60 minutes ($p<0.05$) the positive control group, 50mg/kg polypeptide-k compound, 1ml essential oil and also 2ml essential oil as compared to negative control group. The maximum mean paw volume was observed at 60 minutes after carrrageenan injection (3.00 ± 0.13). At 60 minutes after carrageenan injection, the group treated with 50mg/kg polypeptide-k compound shows higher percentage inhibition of edema (63.29%), followed by 10mg/kg polypeptide-k compound (46.24%), 2ml essential oil administration (41.33%), 1ml essential oil administration (36.13%), and lastly positive control group (5.78%). In the air pouch study, there is significant suppression of total and absolute leukocytes count ($p<0.05$) seen in the treatment groups. Neutrophils and monocytes counts were significantly decreased ($p<0.05$) in treatment groups except in 10mg/kg polypeptide-k group. The group treated with 1ml essential oil shows higher percentage inhibition of leukocytes count (69.76%) when compared with negative control group followed by 50mg/kg polypeptide-k compound (63.95%), 2ml essential oil (62.81%), 10mg/kg polypeptide-k compound (49.32%) and lastly positive control group (48.89%). There is

significant decrease ($p<0.05$) of nitrite concentration in positive control and 50mg/kg polypeptide-k groups. The concentration of nitrite in negative group is 0.25 ± 0.049μmol/gram which is significantly higher ($p<0.05$) than other treated group. Meanwhile for the interleukin 10 (IL-10) concentrations in all treated groups were not significantly different from control. The outcome of this study indicates that the polypeptide-k compound and essential oil extracted from *Momordica charantia* seeds having potential in inhibition of paw edema volume and also have the ability to inhibit acute inflammation by reducing the total leukocytes count and production of nitrite. When compared between polypeptide-k compound and essential oil extraction, it shows that 50mg/kg polypeptide-k compound is more potent that essential oil in inhibiting acute inflammation in rats.

KESAN SEBATIAN POLIPEPTIDA-K DAN MINYAK ESENSIAL DARIPADA BIJI *Momordica charantia* KEATAS INFLAMASI AKUT RANGSANGAN DARIDAPA KARAGENAN

ATIKAH KAZIMAH BT AHMAD

ABSTRAK

Inflamasi merupakan salah satu tindakbalas badan terhadap jangkitan dan kecederaan. Terdapat laporan menyatakan ubat anti-inflamasi akan memberi kesan sampingan kepada manusia. *Momordica charantia* merupakan salah satu contoh herba tradisional yang berkebolehan merawat inflamasi. Tujuan kajian ini adalah untuk mengenalpasti kesan anti-inflamasi sebatian polipeptida-K dan minyak esensial daripada biji *Momordica charantia* dalam inflamasi akut rangsangan daripada karagenan. Aktiviti anti-inflamasi yang ditunjukkan oleh sebatian polipeptida-K dan minyak esensial diukur dengan menggunakan model pembengkakan kaki dan model kantung udara. Dalam kajian ini, 36 ekor tikus *Sprague dawley* dibahagikan kepada enam kumpulan yang mengandungi 6 ekor tikus setiap kumpulan. Kumpulan 1 menerima 5ml/kg air suling, kumpulan 2 menerima 10mg/kg indomethacin, kumpulan 3 dan 4 menerima 10mg/kg dan 50mg/kg sebatian polipeptida-K manakala kumpulan 5 dan 6 menerima 1ml dan 2ml minyak esensial. Setiap ekor tikus diberikan rawatan 3 hari sebelum suntikan karagenan. Dalam model pembengkakan kaki, rawatan terakhir diberi pada hari keempat iaitu 30 minit sebelum 0.1ml karagenan disuntik ke dalam tapak kaki belakang setiap tikus. Isipadu tapak kaki diukur dengan menggunakan 'plethysmometer' sebelum dan setiap jam selepas suntikan diberi. Manakala dalam model kantung udara, suntikan udara steril dimasukkan ke bahagian belakang tikus. Pada hari keempat, tikus menerima rawatan mengikut kumpulan masing-masing. Karagenan disuntik ke dalam kantung udara untuk menghasilkan kesan inflamasi akut. Selepas enam jam, kantung udara dibasuh dan cecair dikumpul untuk mengenalpasti isipadu cecair, jumlah sel darah putih, interleukin 10 (IL-10), dan penghasilan nitrite. Dalam model pembengkakan kaki, terdapat penurunan signifikan oleh isipadu kaki pada minit ke 60 dalam kumpulan 2, kumpulan 50mg/kg sebatian polipeptida-K, kumpulan 1ml dan kumpulan 2ml minyak esensial. Isipadu kaki tikus mencatatkan bacaan tertinggi bagi kumpulan 1 selepas karagenan disuntik (0.83 ± 0.1085). selepas 60 minit karagenan dimasukkan, kadar penurunan isipadu tapak kaki bagi kumpulan 50mg/kg polipeptida-K mencatatkan bacaan tertinggi iaitu 74.75% diikuti oleh kumpulan 10mg/kg polipeptida-K (63.02%), kumpulan 2ml minyak esensial (59.64%), kumpulan 1ml minyak esensial (56.06%) dan akhir sekali kumpulan 2 iaitu 31.21%. Dalam model kantung udara, terdapat penurunan signifikan sel darah putih diantara kumpulan. Juga terdapat penurunan signifikan bagi sel neutrofil dan monosit dalam kesemua kumpulan kecuali kumpulan 10mg/kg polipeptida-K. Kumpulan 1ml minyak esensial mencatatkan kadar penurunan sel darah putih yang tertinggi jika dibandingkan dengan kumpulan 1 iaitu sebanyak 69.76%, diikuti dengan kumpulan 50mg/kg polipeptida-K (63.95%), kumpulan 2ml minyak esensial (62.81%), kumpulan 10mg/kg polipeptida-K (49.32%) dan akhir sekali

kumpulan 2 iaitu 48.89%. Terdapat penurunan signifikan bagi penghasilan nitrite dalam kumpulan 2 dan kumpulan 50mg/kg polipeptida-K. Manakala tiada perubahan signifikan bagi penghasilan IL-10 dalam kesemua kumpulan rawatan. Berdasarkan keputusan yang diperolehi, dipercayai bahawa sebatian polipeptida-K dan minyak esensial daripada biji *Momordica charantia* dapat mengurangkan inflamasi akut. 50mg/kg polipeptida-K dipercayai dapat mengurangkan isipadu tapak kaki tikus dengan mengurangkan kemasukan cecair diawal peringkat inflamasi. Manakala minyak esensial dipercayai dapat mengurangkan kemasukan sel darah putih ke kawasan inflamasi diakhir peringkat inflamasi akut.

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APPROVAL

I hereby certified that I have read this project paper entitled "In-vivo Anti-Inflammatory Studies of Polypeptide-K and Essential Oil from *Momordica charantia* Seeds (Bitter Gourd)" by Atikah Kazimah bt Ahmad, and in my opinion it is satisfactory in terms of scope, quality and presentation as a fulfillment of the requirement for SBP 3999 course.

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DECLARATION

I hereby declare that this thesis is based on my original work except for quotation and citation which has been acknowledged.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

NSAID	Nonsteroidal Anti-inflammatory Drug
NO	Nitric oxide
NOS	Nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
iNOS	Inducible-type nitric oxide synthase
COX	Cyclooxygenase
NED	Napthylethylenediamine dihydrochloride
IL-10	Interleukin 10
IL-2	Interleukin 2
IFN-γ	Interferon gamma
TNF-α	Tumor necrosis factor alpha
NaCl	Sodium Chloride
S.E.M	Standard Error of Mean
mg	milligram
kg	kilogram
ml	milliliter
nm	nanometer
%	percentage
<	less than
>	more than

CHAPTER I

INTRODUCTION

1.1 BACKGROUND

Inflammation is one of the most important and popular disease affecting the world's population. Inflammation occurs in tissue that is injured or disturbed that usually because of the external factors. This process is a protective attempt by the immune response or body defenses in our body to remove the injurious stimuli as well as initiate the healing process for the tissue. However, the untreated inflammation can lead to chronic inflammation such as rheumatoid arthritis, skin diseases such as eczemas and psoriasis, some intestinal disease and also hay fever. Furthermore inflammation has been linked to many things that associate with aging, including wrinkles, heart diseases, Alzheimer and cancer.

Scientist had developed several types of anti-inflammatory drugs such as steroidal and non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are usually use for the treatment of acute or chronic conditions where inflammation such as pain, swelling and stiffness are present. NSAIDs are classified according to their chemical structure or mechanism of action. Examples of NSAIDs are such as Ibuprofen, Ketoprofen,

Mefenamic Acid and Indomethacin. However, prolong used of NSAIDs can lead to two main adverse drug reactions that related to gastrointestinal effects and renal effects. Symptoms that related to gastrointestinal effects are mucosal erosions that can progress into ulcers and life-threatening complications such as perforation and hemorrhage (Loren, 2003). Due to adverse effects that associated with the consumption of NSAIDs, scientist had think of development of new anti-inflammatory agents with minimum side effects such as agents that had been made from natural compound.

Medicinal plants are still a major part of traditional medical systems in developing countries. Several herbal remedies are now being intensively used in therapy. It is also a treatment method, which bases on using traditional herbs which supported by scientific fact. Numerous natural products have been already tested in various animals models for the development of new anti-inflammatory therapeutics (Sharif *et al*., 2010). Scientific study a large number of plant species contain various bioactive compounds exhibiting health beneficial properties and anti-inflammatory effects and their preventive and therapeutic use increases. It has been proven that many herbs or plant extract successfully treat inflammation such as *Lippia gracilis* leaves (Mendes *et al*., 2010), essential oil and active compound from *Cordia verbenacea* (Giselle *et al*., 2007), and also extract of *Angelica gigas* (Shin *et al*., 2009).

Momordica charantia is also known as bitter melon or bitter gourd. They grows in tropical areas, including parts of the Amazon, cast Africa, Asia and the Caribbean and is cultivated throughout South America as a food and medicine. Mostly local people in the

Amazon used bitter melon's fruit and leaves in their preparation of food. Furthermore, a leaf tea is used for diabetes, to expel intestinal gas, to promote menstruation and antiviral. Meanwhile in Brazilian herbal medicine, bitter melon is used for tumors, wounds, rheumatisms, inflammation and diabetes. Studies had shown that bitter melon fruit has properties of lowering blood glucose, moreover it shown the ability to enhance cell uptake of glucose to promote insulin release (Chun *et al*., 2008). The fruits also had been discovered having anti-ulcerogenic effects on the ethanol-induced ulcerogenesis model in rats (Ilhan *et al*., 2000). Meanwhile the seeds extract had shown broad-spectrum antimicrobial activity (Galberto *et al*., 2010). *Momordica charantia* contains an array of biologically active phytochemicals include triterpenes, proteins and steroids. It has been traditionally used as medicinal herbs as anti-inflammatory, anti-leukemic and anti-tumor (Jesada *et al*., 2005). The further investigation on *Momordica charantia* in treating inflammatory conditions should be continued.

1.2 OBJECTIVE

GENERAL OBJECTIVE

- To study the effects of Polypeptide K compound and essential oil from *Momordica charantia* seeds on induced inflammation in experimental rats.

SPECIFIC OBJECTIVES

1. To determine the effect on carrageenan-induced paw edema by measuring the paw edema volume.
2. To determine the effect on carrageenan air pouch by determine the exudates volume, total and differential leukocyte count.
3. To determine the release of interleukin 10 (IL-10) from supernatant that had been collected in air pouch.
4. To determine the nitric oxide (nitrite) concentration from the supernatant that had been collected in air pouch.

CHAPTER II

LITERATURE REVIEW

2.1 *Momordica charantia*

Momordica charantia (Cucurbitaceae) is also known as bitter gourd, bitter melon or Chinese bitter melon. The leaves and fruits are both used for the making of beer and teas, sometimes in making of soup usually in the Western world. The fruits are suitable to be eaten when harvested green and cooked. The tastes are bitter. *Momordica charantia* is like a green cucumber shaped fruit with gourd-like bumps all over it. It looks like an ugly, light green cucumber and the fruit should be firm (Figure 2.1). The stems which are ridged have small hair on it, while the first leaves are unlobed and heart shape with a series of broad teeth along the margin. These palmate leaves 10-12cm wide with hairy to smooth surfaces. Almost whole parts of *Momordica charantia* such as seeds, leaves and vines have their own beneficial but the fruit is the safest and most prevalent part of the plant used medicinally (Grover *et al*., 2004).

2.1.1 Habitat and cultivation

Momordica charantia grow in tropical areas of Asia, Amazon, East Africa and the Caribbean. It is also known as Karela and Balsam Pear among Indian people. It is cultivated all around the world as vegetables and medicine (Grover *et al*., 2004). This plant can be found in the wild and it able to grow in dry and moist areas. This plant may also grow in a temperature range of 12.5°C to 25°C with soil pH from 4.3 to 8.7, where annual rainfall may be as low as 480mm to as much as 4100mm. *Momordica charantia* can also be found in south eastern United State of America as well as tropical countries in South East Asia such as Malaysia, Indonesia, Java and China. In Malaysia almost every state cultivated this plant and used it in preparation of meal and as vegetables.

2.1.2 Therapeutic usage

Momordica charantia has astringent properties and being purgative herbs that can reduce inflammation as well. The astringent properties of bitter gourd also cause hypoglycemic effects (Chun *et al*., 2008). In Peruvian herbal medicine, the leaves or aerial parts of the plant are used to treat measles, malaria and all types of inflammation. Meanwhile in Nicaragua, the leaf is used for stomach pain, diabetes, fever, coughs, headache and infection. Furthermore, according to ayurvedic practitioners, the fruits have properties of cooling, digestive, laxatives, antipyretic and appetite stimulant. The

leaves extract also increased resistance to viral infections and had immunostimulant effects in humans and animals by increasing interferon production and natural killer cells activity (Basch *et al*., 2003). The essential oil of the seeds of *Momordica charantia* has antibacterial properties by inhibited the growth of *Staphylococcus aureus* (Barca *et al*., 2008). Polypeptide K compound that had been extracted from the *Momordica charatia* seeds has anti diabetic properties by reducing the range of blood sugar level (Khanna *et al*., 2008).

The leaves, flowers and fruits of *Momordica charantia* contain alkaloids called momordicine. Momordicine is the most important chemical makeup of bitter melon and that is making the bitter gourd taste bitter. Other chemical compounds that can be found include bitter glucoside, resin, aromatic oil and substances similar to saponin and mucilage. There are about twenty five compounds that had been isolated in the *Momordica charantia* seeds such as susquiterpenes, phenylpropanoids, monoterpenes and more (Braca *et al*., 2008). Momordicatin compound that had been extract from *Momordica charantia* by aqueous extraction have potential in developing new chemotherapeutics against leishmaniasis (Shreedhara *et al*., 2010).

Figure 2.1: *Momordica charantia*

2.2 Inflammation

Inflammation is a physiological response of body to stimuli including infections and tissue injury. Inflammatory response protects the host against injury and microbial invasion. The body response should be in short-lived (Yu Tang *et al*., 2008). However, excessive or prolong inflammation can cause a variety of pathological conditions such as bacterial sepsis, rheumatoid arthritis and skin inflammation (Sharif *et al*., 2010). It is a process by which the white blood cells and chemicals protect us from infection and foreign substances such as bacteria and viruses. Inflammation also can occur when the body's defense system or immune system has inappropriately triggered an inflammatory response when there are no foreign substances to fight off and it is called autoimmune diseases (Arazi *et al*., 2010).

2.2.1 Cardinal Signs of Inflammation

Inflammation usually is a localized event. It produces localized symptoms such as swelling, redness, heat, pain and loss of function. Swelling is due to the accumulation of fluid in the tissue, redness is due to increased blood flow to the area, heat is also because of the blood flow to the area of injury, and pain is due to the action of bradykinin and prostaglandins on the local nerve tissues (Ruslan, 2010).

2.3 Acute inflammation

Inflammation can be divided into two categories such as acute inflammation and chronic inflammation. Acute inflammation begins within seconds to minutes after the injury of tissues. It is the early phase of response that involves polymorphonuclear neutrophils leukocytes as the principles cellular effectors. Acute inflammatory response has three basic stages which is vasodilation and increased permeability of blood vessels, emigration of phagocytes from the blood into interstitial fluid and tissue repair (Tortora and Derrickson, 2006). Vasodilation is due to increased of blood flow to supply to the injured region. The capillaries increased in permeability to allow the fluid and blood protein to move to interstitial spaces and migration of neutrophils act to remove the foreign substances. The acute inflammatory reaction also characterized by exudation of fluid and plasma proteins that lead to local edema formation consist of leukocyte-dependent and leukocyte-independent component (Lazzarani *et al*., 2006; Garcia-Lerne, 1989).

According to Robbins and Cotran (2006), acute inflammation can be characterized by vascular changes and cellular events.

Vascular Changes

During injury, the vascular wall changes in their permeability and caliber that affect the flow. These changes occur at various rates depending on severity of the injury. Vasodilation causes increased flow into the areas of injury therefore increased in

hydrostatic pressure. Meanwhile increased in vascular permeability causes infiltration of protein rich fluid and decreased plasma osmotic pressure. The combination of increased hydrostatic pressure and decreased osmotic pressure cause net outflow of fluid and edema formation. Inflammatory mediators released lead to vascular changes that act mainly by increasing the microvascular permeability to macromolecules in the post capillary venues that enhancing plasma protein efflux (Lazzarani *et al*., 2006; Yong and Mayhan, 1992). Increased in vascular permeability will result in increased local blood flow.

Cellular event by leukocyte extravasation and phagocytosis

Vascular permeability will lead to leukocyte delivery to the sites of injury. This event is called extravasation which involves three steps:

- Margination, rolling and adhesion of leukocytes to the endothelium.
- Transmigration across the endothelium.
- Migration in interstitial tissues toward chemotactic stimulus.

2.3.1 Carrageenan-induced acute inflammation

Carrageenan is actually gel-like substances that act as foreign materials that can produce inflammatory response. Carrageenan-induced inflammation is acute, nonimmune and highly reproducible (Morris, 2003). Acute inflammatory response is

characterized by increased vascular permeability, extravasation of fluid and leukocyte infiltration. Release of chemical mediators such as histamine, serotonin and bradykinin occurs in the initial phase of inflammation at 0 to 1 hour, whereas release of prostaglandins is at the late phase which is at 1 to 6 hour (Emanuela *et al*., 2005; Di Rosa *et al*., 1971; Vinegar *et al*., 1969). Prostaglandin contributes to the induction of cyclooxygenase-2 (COX-2) in the inflamed tissue (Nantel *et al*., 1999). There is also release of nitric oxide as the chemical mediator that involve in the ability to increase vascular permeability and edema through changes in local blood flow (Emanuela *et al*., 2005; Moncada *et al*., 1993).

2.3.1.1 Carrageenan-induced paw edema

Carrageenan was firstly described by Winter *et al*., (1962). Once carrageenan is injected into the hind paw, there will be a release of chemical mediators such as bradykinin, serotonin and histamine followed by release of prostaglandin and nitric oxide (Giselle *et al*., 2007). These agents can be generated in situ at the site of insult or by infiltrating cells. Once injected, the paw volume increase maximal around 5 hour and modulated by inhibitors of specific molecular within the inflammatory cascade (Morris, 2003). In carrageenan-induced paw edema, it consists of nonphagocytic inflammatory response followed by phagocytic inflammatory response (Vinegar *et al*., 1987). Nonphagocytic respond consisted of edema, hyperemia and hyperalgesia meanwhile phagocytic inflammatory response comprised of mobilization of neutrophils, edema,

hyperalgesia, mobilization of monocytes and proliferation of fibroblast and vascular tissue.

2.3.1.2 Canatoxin-induced paw edema

Canatoxin-induced paw edema was observed in two phases. The first phase, there was increase in paw volume between 0 to 2 hour after canatoxin was injected with no involvement of inflammatory phagocytic cells. Meanwhile second phase started at third hour that characterized by cellular infiltration at the site of injection which lead to further increase in paw swelling. They observed that the volume maximum at six hour after canatoxin injected and usually disappeared within 48 hour at low doses of canatoxin. The chemical mediators that likely involved in first phase were such as histamine, serotonin, PAF and prostaglandins meanwhile, in second phase, leukotrienes play major role in the development of cellular infiltration at the inflammatory site (Benjamin *et al*., 1992).

2.3.1.2 *Bothrops lanceolatus* venom-induced paw edema

There are two types of administration of *Bothrops lanceolatus* venom. The first one is by non-heated and heated venom. The non-heated produce intense haemorrhage meanwhile heated with no haemorrhage edema. The paw volume was maximal within 15 minutes and disappearing over 24 hour. *Bothrops lanceolatus* venom-induced paw

edema mediated by kinins and lipoxygenase metabolites that involves mast cell degranulation that cause the release of histamine and serotonin (L.de Faria *et al*., 2001).

2.3.1.3 Dextran-induced paw edema

Dextran induced edema is a consequence of liberation of histamine and serotonin from mast cells (Chawla *et al*., 1987). After 30 minutes injection of dextran, the paw volume was measured in 4 hour duration time (Okunrobo *et al*., 2009).

2.3.1.4 Carrageenan-induced air pouch

Air pouch is a well-establish model in inflammation. It consists of a subcutaneous injection of air on the back of the animal usually rodents. Once air is injected, there will be proliferation of cells into the pouches which is similar to infiltration of synovial fluid after 6 days (Nasrin *et al*., 2010; Edward *et al*., 1981). Inflammation is induced by carrageenan injection into the pouches where it serves as a reservoir of cells and mediator. Inflammatory cells and mediators can be easily measured in the fluid because it accumulates locally (Nasrin *et al*., 2010). This model has some advantage where there is no involvement of internal organs which can be damaged during sampling (Martin *et al*., 1994; Sedgwick and Lees, 1986).

2.4 Nonsteroidal Anti-inflammatory Drugs (NSAIDs)

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most prescribed medications for treating inflammation diseases. They help in reducing inflammation and lower fevers. Examples of NSAIDs are Ibupofen, Ketoprofen, Indomethacin and Mefenamic acid. NSAIDs block the COX enzymes and reduce prostaglandins which promote inflammation, pain and fever. Unfortunately only COX-1 produces prostaglandins that support platelets and protect the stomach. So prolong uses of NSAIDs can cause ulcers in the stomach and promote bleeding (Loren, 2003).

2.5 Nitric Oxide (NO)

Nitric oxide (NO) synthesized by nitric oxide synthase (NOS) which is involved in physiological processes. Unfortunately increase in NO production will lead to various inflammatory diseases such as sepsis, arthritis, multiple sclerosis, and systemic lupus erythromatosis (SLE). There are three isoforms of NOS depend to the physical and biochemical characteristics of the purified enzymes. Type I is neuronal NOS (nNOS), type III is endothelial NOS (eNOS) and type II is an inducible-type NOS (iNOS). Activation of iNOS pathway is after it had been exposed to specific stimulants in some cells such as cytokines, bacterial polysaccharides and calcium ionophores to induce inflammatory response (Denlinger *et al*., 1996). The expression and activity of iNOS is the major therapeutic target to treat various inflammatory responses (Lee *et al*., 2007).

Carrageenan is one of the examples of inflammatory agent that can lead to activation of iNOS pathway.

Nitric oxide is produced by many cells in the body but production of nitric oxide in inflammation is classified as defense mechanism of the body against microorganism by activating of macrophages and neutrophils. The production by the vascular endothelium is important in the regulation of blood flow (Csaba, 2000). Increase in nitric oxide production will lead to increase endothelial permeability. Detection of nitric oxide itself was difficult because there are rapidly metabolized to the stable end product which is nitrite and nitrate (Moshage, 1997). Nitrite can be detected using Griess reagent where the principle behind it is by the chemical reaction uses sulfanilamide and napthylethylenediamine dihydrochloride (NED). Under acidic conditions, sulfanilamide and NED will compete for nitrite in Griess reaction (Moshage, 1997).

2.6 Interleukin 10 (IL-10)

IL-10 is an anti-inflammatory cytokine that had been secreted by various cells such as activated CD4+ and CD8+ T cells, macrophages, natural killer cells and B cells. The physiological response of IL-10 is to regulate macrophages activated by pathogens and their product (Peter, 2006). IL-10 is known to be major regulator in suppressing the inflammatory response. IL-10 inhibits pro-inflammatory cytokine production such as TNF-α, IL-1, IL-12 and IFN-γ by regulating immunomediated inflammation (Fiorentino *et al*., 1991). IL-10 inhibited the infiltration of neutrophils and macrophages towards the

inflammation site. In addition it also inhibited over expression of chemokines and macrophage inflammatory (Yasunori *et al*., 1999). In vitro studies of transgenic mice shown the important of IL-10 in controlling inflammation initiated and continue by pro-inflammatory signals in acute and chronic diseases (Peter, 2006; Moore *et al*., 2001).

CHAPTER III

MATERIALS AND METHOD

3.1 Chemical and Drug

Polypeptide-K compound and essential oil (Magna Mission SDN BHD, Malaysia), 500ml of 0.9% Normal Saline, 0.1ml of 1% Carrageenan (Sigma Co. Ltd, Malaysia), 5ml of 0.5% Carrageenan (Sigma Co. Ltd, Malaysia), Indomethacin (Sigma Co. Ltd, Malaysia), Griess' Reagent, Rat IL-10 platinum ELISA (eBioscience Co, San Diego), Sodium Nitrate (Sigma Co. Ltd, Malaysia), Sodium Chloride (Sigma Co. Ltd, Malaysia), 25 G sized and 27 G sized surgical needle and syringe.

3.2 Experimental Animals

36 male *Sprague Dawley* rats with weight in range of 250g-300g were used in this study. All 36 rats were randomly divided into six different groups according to different types of treatment. All of the rats were place in the Animal House of Faculty of Medicine and Health Sciences, University Putra Malaysia. The animals undergo adaptation period for about 2 weeks before treatment was given. The rats were fed with standard rat pellet and water.

3.3 Experimental Design

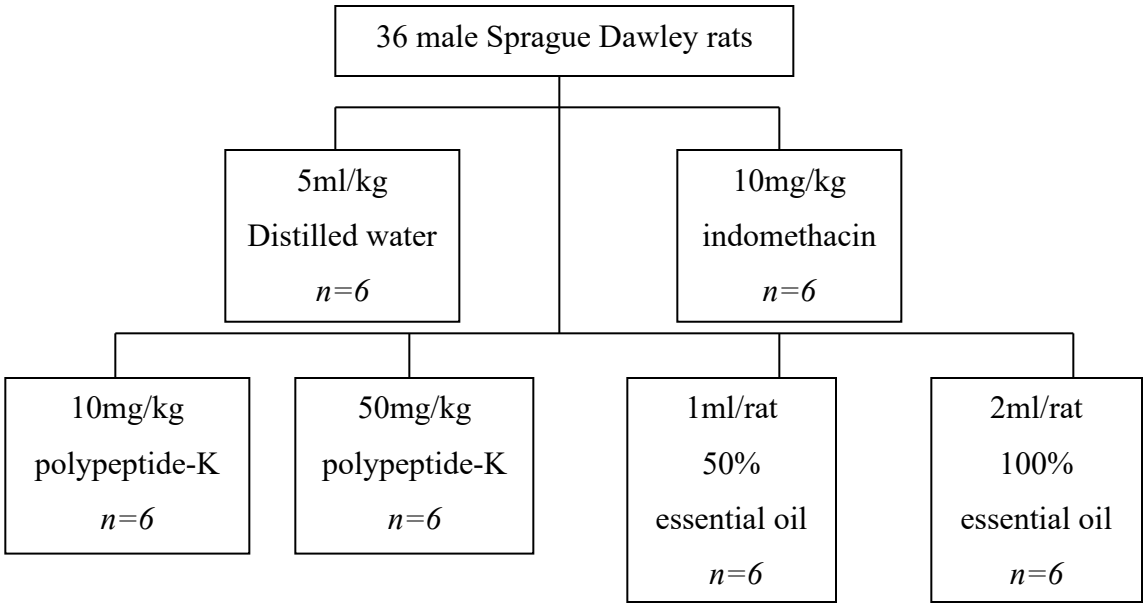


Figure 3.1: Experimental design with different types of treatment

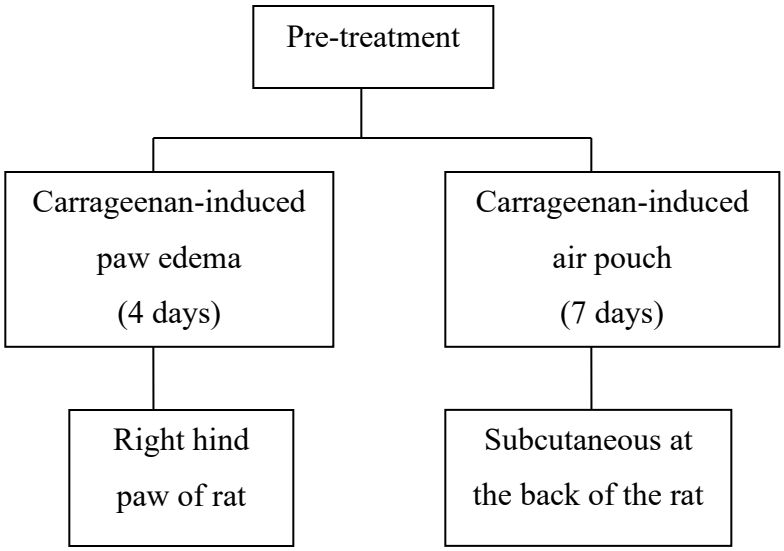


Figure 3.2: Experimental design of carrageenan-induced paw edema and carrageenan-induced air pouch

According to figure 3.1, all 36 rats will be divided into 6 groups. Group 1 had received 5mg/kg distilled water with the mixture of 1% DMSO as vehicle. Meanwhile group 2 had received 10mg/kg indomethacin as positive control. Group 3 and 4 had been given 50% and 100% essential oil respectively and meanwhile group 5 and 6 received 10mg/kg and 50mg/kg polypeptide-K respectively. All treatments were given orally through a force-feed needle fixed to a syringe.

3.4 Carrageenan-induced Paw Edema in Rat

36 male *Sprague Dawley* rats with weight in the range of 250g to 300g were used in this study. All rats in each group were given treatment orally for duration of four days. Group 1 received distilled water, group 2 received 10mg/kg indomethacin. Group 3 and 4 were given 50% and 100% essential oil respectively whereas group 5 and 6 received 10mg/kg and 50mg/kg polypeptide-K compound respectively.

On the fourth day, the final oral administration of each treatment was given 30 minutes before carrageenan injection at the right hind paw. The pletysmometer which was used to measure the displacement volume of the paw of each rat was calibrated using standards (Olajide *et al*., 2000).

Volume of the right hind paw was measured before the injection of carrageenan. Then 1ml of lambda carrageenan was injected subcutaneously into the right hind paw of each rat and the volume was measured immediately prior to injection. The measurement was taken continuously every 30 minutes for duration of 5 hours. The inhibitory activity was calculated according to the following formula (Cheng, 2007).

Percentage inhibition

$$= \frac{(Ct - Co)\ \text{control} - (Ct - Co)}{(Ct - Co)\ \text{control}} \times 100$$

$$= \frac{\Delta k - \Delta e}{\Delta k} \times 100$$

Where Δk = difference in paw weight in the control group; and Δe = difference in the paw weight in the treatment group.

3.5 Carrageenan-air pouch model

Rats were divided into six groups (*n=6*) and were administered with distilled water for group 1 as negative control. Group 2 received 10mg/kg indomethacin as positive control. Meanwhile group 3 and group 4 received 50% essential oil and 100% essential oil respectively. Lastly group 5 and group 6 administered with 10mg/kg and 50mg/kg polypeptide-K compound respectively. On day 1, the rats had been anesthetized with diethyl ether, and then 20ml of sterile air were injected subcutaneously on their back to form pouches. On day 4, the rats were received pretreatment according to their group. Right after administration of treatment, the rats were again anesthetized with diethyl ether and the pouches that had been form on day 1 were re-injected with another 20ml of sterile air. This technique called re-inflated to make sure that the pouches are well preserved. All rats in each group were given treatment orally for

duration of four days. On day 7, 30 minutes after oral administration of treatment, 5ml of 0.5% carrageenan were injected into the pouch. After the injection of carrageenan, the back of the rats were massage gently to make sure that the carrageenan are well distributed in the pouch. After 6 hours, the rats were euthanized with chloroform. Then the pouch was washed with 10ml normal saline. The exudates was immediately collected and cooled on iced and the volume was recorded (Shin *et al*., 2009). The exudates volume was determined as the net volume from which the initially injected volume of saline was subtracted. The collection of exudates was then centrifuge at 4900rpm for 10 minutes in 4°C. Then the supernatant was used for the detection of interleukin-10 and nitric oxide. Meanwhile the sediment was mixed with 2ml of saline for total cell count using automated cell count machine and slide smear for the differential cell count.

3.6 Measurement of Interleukin-10

After centrifuge the exudates, the supernatant was used for the detection of interleukin-10. The detection of interleukin-10 is by using rat IL-10 platinum ELISA that had been provided by eBioscience company (Refer to appendix).

3.7 Determination of Nitrite from Carrageenan-induced air pouch model in rat

Rats were divided into six groups (*n=6*) and were administered with 5ml/kg of distilled water, 10mg/kg of Indomethacin, 10mg/kg and 50mg/kg polypeptide-K

compound for group 3 and 4 respectively, lastly 50% and 100% essential oil for group 5 and 6 respectively. On the seventh day, which is the final oral administration of each treatment was given 30 minutes before carrageenan injection for the air pouch model rats. After 6 hours, the exudates were collected and centrifuge at 4900rpm for 10 minutes in 4°C. 50µl of supernatant was transfer to a 96-well microplate in duplicate, followed by 50µl of Griess reagent. After incubation for 10 minutes at room temperature, the color of the product dye developed and its absorbance was detected at 548nm by ELISA microplate reader. The calibration curve was made with sodium nitrite in distilled water (linear range 0-100µM) refer to appendixes.

3.8 Automated Cell Count and Differential Cell Count

After the collection of exudates from the preserved pouch, the exudates were centrifuge at 4900rpm for 10 minutes in 4°C. After centrifuge, the sediment was mixed with 2ml saline for the automated cell count by using automated hematology analyzer in Hematology Lab. Meanwhile one drop of sediment mixture was used for differential cell count by smear it on a slide. The differential count was performed by counting and identifying at least 100 leukocytes. The absolute leukocyte count is calculated by multiplying the percentage of each cell type such as neutrophils, monocytes and lymphocytes in differential count with total white blood cell count formed in exudates.

3.9 Statistical analysis

All data were obtained and analyzed using statistical software. The test performed is one-way analysis of variance and was further analyzed using the Tukey post hoc Test. Value of $p<0.05$ were considered as significant.

CHAPTER IV

RESULT

4.1 Carrageenan- induced Paw Edema in Rat

In Carrageenan-induced paw edema, there was a gradual increase in the edema volume in the negative control group. The maximum mean paw volume was observed at 60 minutes after carrrageenan injection (0.8383 ± 0.10849). However, the paw volume begins to decrease for the following hours till 180 minutes after injection but increase again at the second peak at 210 minutes after carrageenan injection. There is significant reduction of paw edema volume at 60 minutes ($p<0.05$) when compare to negative control group for the positive control group, 50mg/kg polypeptide-K compound, 1ml essential oil and also 2ml essential oil. Except for 10mg/kg polypeptide-K compound, there is no significant different in the reduction of paw edema volume ($p>0.05$). Following carrageenan injection (0 hr), the mean paw volume for negative control group and positive control group was 0.4650 ± 0.0963, 0.3667 ± 0.1384 respectively. Meanwhile in treatment group of 10mg/kg polypeptide-K and 50mg/kg polypeptide-K group the volume was 0.2426 ± 0.0915 and 0.3250 ± 0.07173. 1ml essential oil was 0.2433 ± 0.1108 and 2ml essential oil was 0.5033 ± 0.1976. The maximum mean paw volume of positive control group was observed at 60 minutes after injection

(0.5767±0.16128) meanwhile 1ml essential oil was observed at 30 minutes after carrageenan injection (0.4050±0.12307). The edema volume begins to decrease for the following hours except for 50mg/kg polypeptide-K compound which had begins to decrease after 30 minutes after injection of carrageenan but slightly increase at 150 minutes. For the 2ml essential oil group, the maximum mean paw volume was observed immediately after the carrageenan injection (0.5033±0.19761), meanwhile for the 10mg/kg polypeptide-K compound the maximum mean paw volume was at 60 minutes after injection (0.2583±0.11347) and start to decrease at the following hours but slightly increase at 300 minutes after injection (0.3567±0.12352). At 60 minutes after carrageenan injection, the group treated with 50mg/kg polypeptide-K compound shows the highest percentage inhibition of edema (74.75%), followed by 10mg/kg polypeptide-K compound (63.02%), 2ml essential oil administration (59.64%), 1ml essential oil administration (56.06%), and lastly positive control group (31.21%).

Group of Treatment	Mean Paw Edema Volume (Mean±S.E.M)						
	0 min	30 min	60 min	120 min	180 min	240 min	300 min
Negative control	0.4650± 0.09629	0.7017± 0.09210	0.8383± 0.10849	0.208±3 0.11438	0.2767± 0.12771	0.3550± 0.17489	0.2550± 0.08917
Positive control	0.3667± 0.13836	0.3067± 0.09865	0.5767± 0.16128	0.2667± 0.06637	0.1933± 0.05162	0.0883± 0.07368	0.1317± 0.02892
10mg/kg polypeptide-K	0.2467± 0.09153	0.2317± 0.11998	0.2583± 0.11347	0.1033± 0.04169	0.2033± 0.09945	0.3433± 0.09611	0.3567± 0.12352
50mg/kg polypeptide-K	0.3250± 0.07173	0.3300± 0.10023	0.2117± 0.06426	0.3900± 0.08185	0.3400± 0.09630	0.2100± 0.06481	0.2667± 0.09752
1ml Essential oil	0.2433± 0.11081	0.4050± 0.12307	0.3683± 0.10806	0.3183± 0.16412	0.3350± 0.09394	0.1800± 0.15776	0.3550± 0.13458
2ml Essential oil	0.5033± 0.19761	0.1350± 0.07680	0.3383± 0.09673	0.2450± 0.05445	0.2850± 0.12987	0.2050± 0.11310	0.2850± 0.08156

Table 4.1: Effect of polypeptide-K and essential oil on mean paw edema volume in carrageenan-induced paw edema

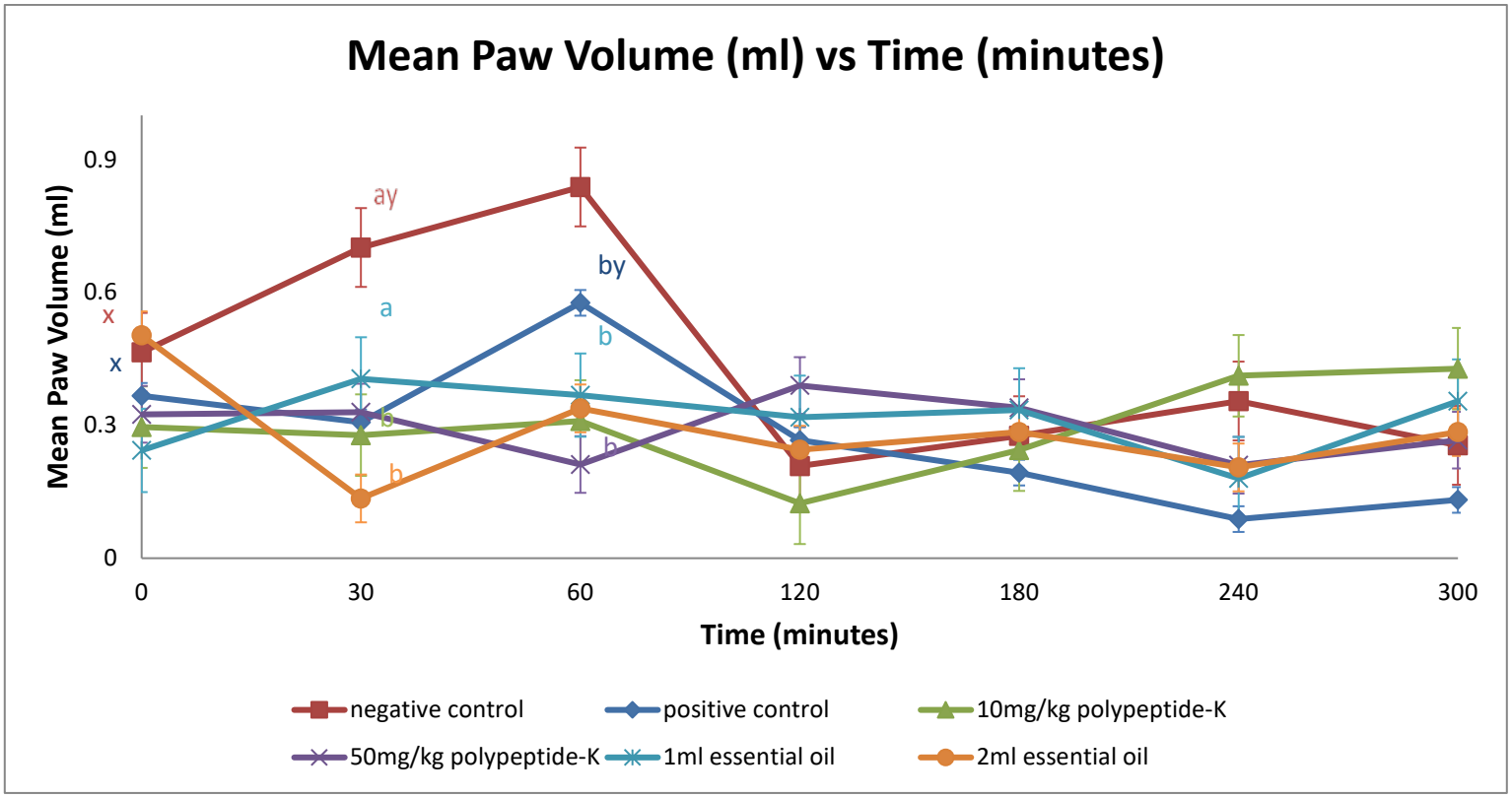


Figure 4.1: Effect of polypeptide-K and essential oil in carrageenan-induced paw edema. Each point represented the mean±S.E.M hind paw volume (ml).

a-b Mean with different superscripts differ significantly ($p<0.05$) between group

x-y Mean with different superscripts differ significantly ($p<0.05$) within group

Group of Treatment	Percentage Inhibition Edema (%)
Negative control (5ml/kg Distilled water)	-
Positive control (10mg/kg Indomethacin)	31.21
10mg/kg polypeptide-K	63.02
50mg/kg polypeptide-K	74.75
1ml per rat essential oil	56.06
2ml per rat essential oil	59.64

Table 4.2: Percentage inhibition edema of polypeptide-K and essential oil on Carrageenan-induced paws edema at 60 minutes after carrageenan injection at the right hind paw

4.2 Carrageenan-induced air pouch

4.2.1 Total Exudates Volume

In air pouch, total exudates volume in negative control group, positive control group, 10mg/kg polypeptide K compound, 50mg/kg polypeptide K compound, 1ml essential oil and 2ml essential oil was 2.013 ± 0.2654, 1.735 ± 0.2186, 1.930 ± 0.3467, 2.433 ± 0.5258, 2.262 ± 0.2554, and 1.940 ± 0.3617 respectively. There is no significant difference ($p<0.05$) of exudates volume between groups.

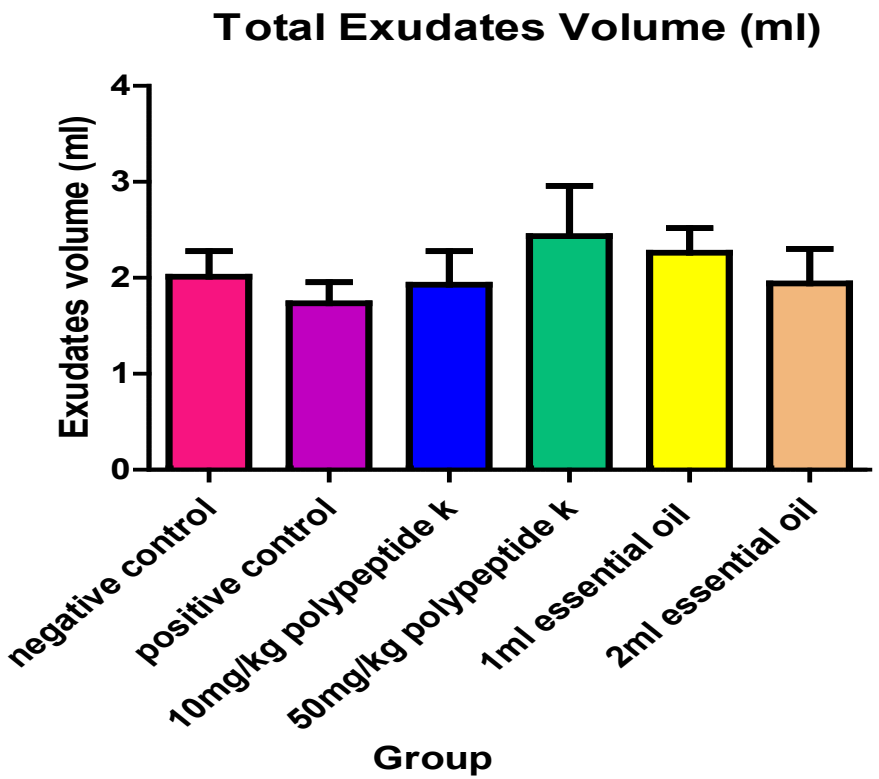


Figure 4.2: Effect of polypeptide-K and essential oil on exudates volume in carrageenan-induced air pouch. The values showed mean±S.E.M exudates volume.

4.2.2 Total Leukocytes Count

In Carrageenan-induced air pouch the mean of total leukocytes count in negative control group, positive control group, 10mg/kg polypeptide-K compound, 50mg/kg polypeptide K compound, 1ml essential oil and 2ml essential oil was 60.967±10.297, 39.067±4.312, 27.225±7.035, 20.967±5.283, and 26.433±4.584 respectively. When compared with negative control group, there is significantly decrease ($p<0.05$) of total leukocytes count in 50mg/kg polypeptide-K compound, 1ml essential oil and 2ml essential oil administration group. Meanwhile for positive control group and 10mg/kg polypeptide-K compound there is no significant decrease ($p>0.05$) of total leukocytes count.

Group of Treatment	**Mean Total Leukocytes Count (Mean±S.E.M)**
Negative control (5ml/kg Distilled water)	60.967±10.297
Positive control (10mg/kg Indomethacin)	39.067±4.312
10mg/kg Polypeptide-K	38.050±6.044
50mg/kg Polypeptide-K	27.225±7.035
1ml per rat Essential oil	20.967±5.283
2ml per rat Essential oil	26.433±4.584

Table 4.3: Effect of polypeptide-K and essential oil on mean total leukocytes count in carrageenan-induced air pouch

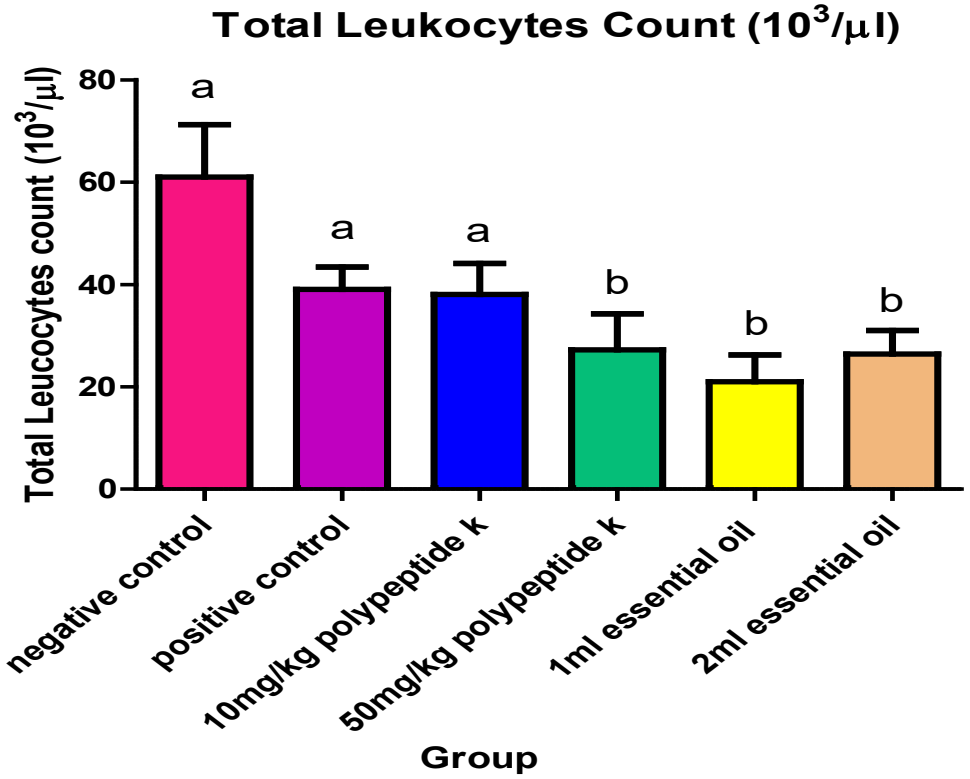


Figure 4.3: Effect of polypeptide-K and essential oil on mean total leukocytes count in carrageenan-induced air pouch. The values showed mean±S.E.M total leukocytes count.

a-b Mean with different superscripts differ significantly ($p<0.05$)

4.2.3 Absolute Leukocytes Count

There is significant difference ($p<0.05$) between the group of treatment in the absolute leukocytes count. Compared to the negative control group, there is significantly decrease ($p<0.05$) in neutrophils count in the treatment groups. Meanwhile for the monocytes count, there is also significant decrease ($p<0.05$) for positive control group, 50mg/kg polypeptide-K compound, 1ml essential oil and 2ml essential oil. Unfortunately for 10mg/kg polypeptide-K group, there is no significantly decrease in monocytes count.

Group of Treatment	**Neutrophils (10^3µl)**	**Monocytes (10^3µl)**
Negative control (5ml/kg Distilled water)	$^{a}43.316\pm8.851$	$^{a}11.590\pm1.816$
Positive control (10mg/kg Indomethacin)	$^{b}22.643\pm2.606$	$^{b}5.4213\pm0.648$
10mg/kg Polypeptide-K	$^{b}20.382\pm3.140$	$^{a}7.4438\pm1.141$
50mg/kg Polypeptide-K	$^{b}15.776\pm4.073$	$^{b}4.0161\pm1.114$
1ml per rat Essential oil	$^{b}12.603\pm3.131$	$^{a}4.0020\pm1.315$
2ml per rat Essential oil	$^{b}14.787\pm2.723$	$^{a}5.6330\pm1.515$

Table 4.4: Effect of polypeptide-K and essential oil on mean absolute leukocytes count in carrageenan-induced air pouch

a-b mean with different superscript differ significantly ($p<0.05$) between groups

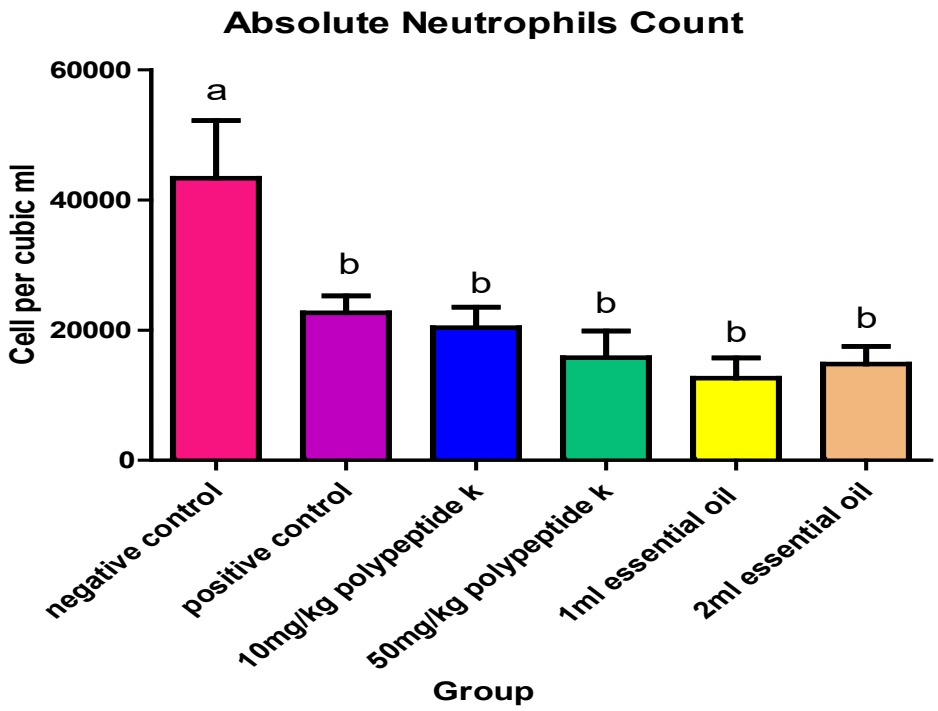


Figure 4.4: Effect of polypeptide-K and essential oil on absolute neutrophils count

a-b mean with different superscript differ significantly ($p<0.05$)

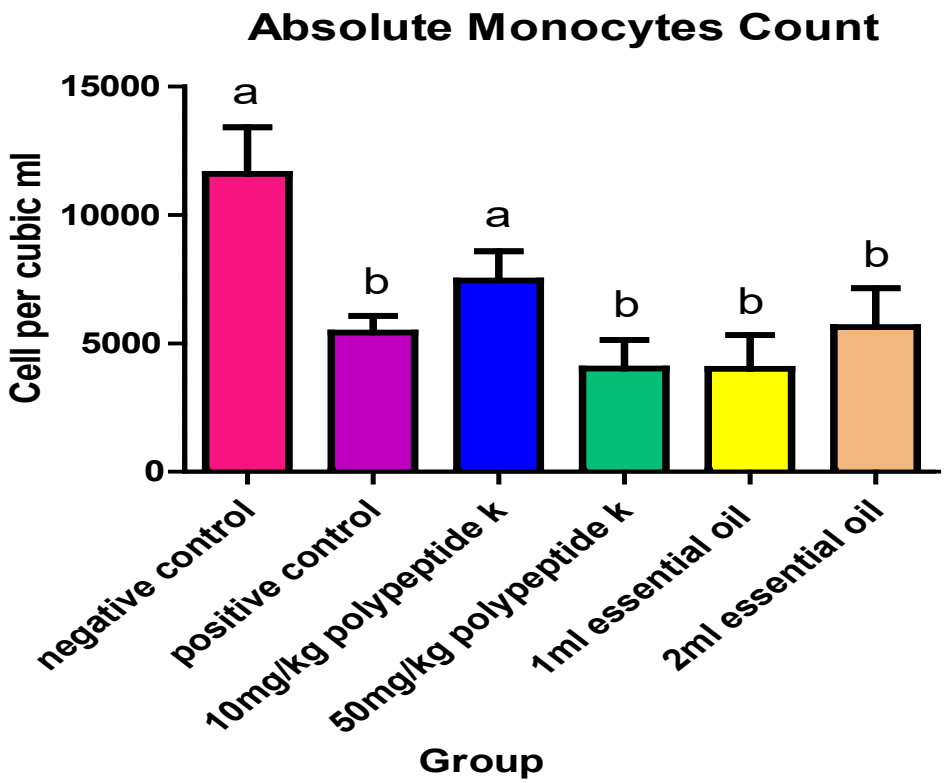


Figure 4.5: Effect of polypeptide-K and essential oil on absolute monocytes count

a-b mean with different superscript differ significantly ($p<0.05$)

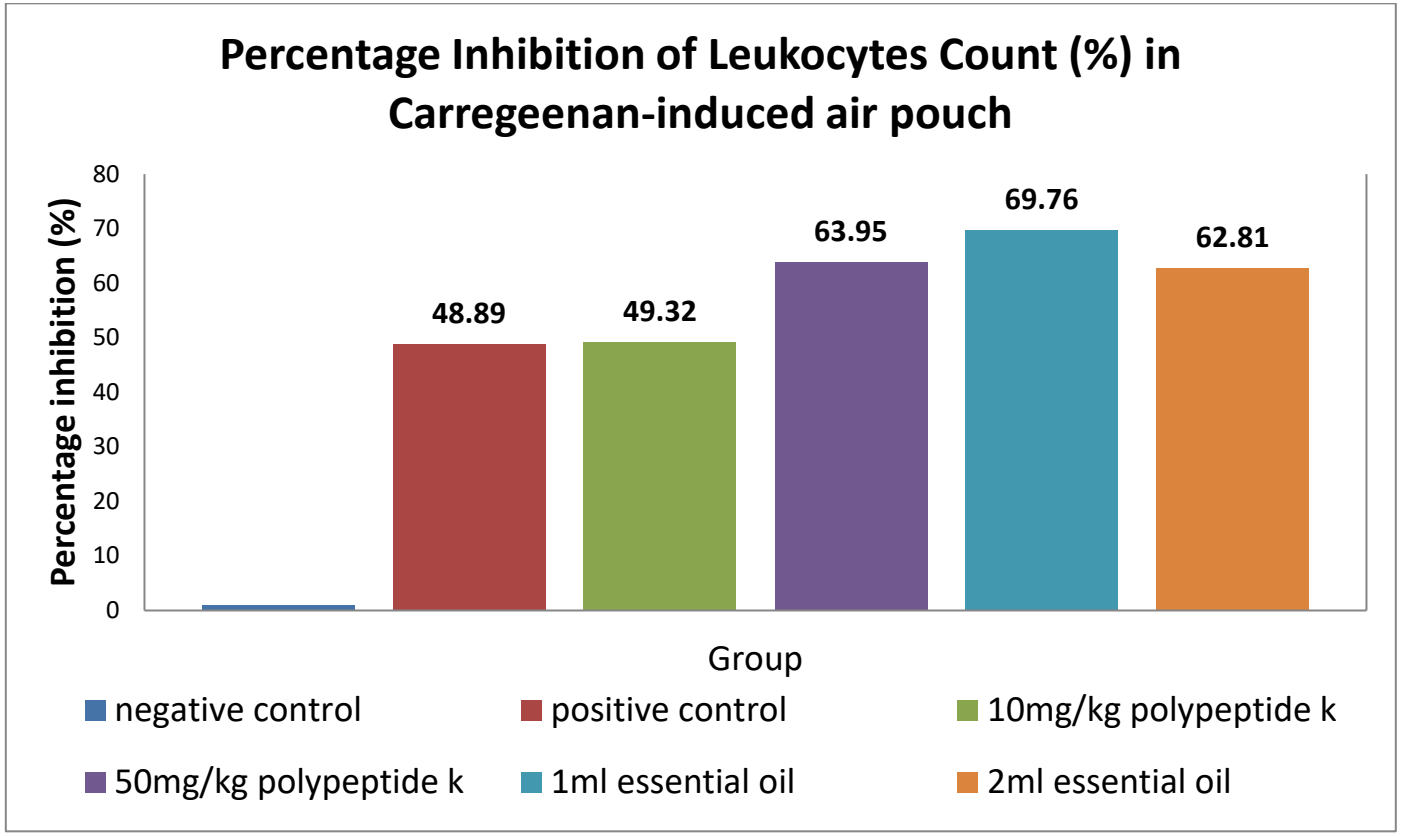


Figure 4.6: Percentage inhibition of leukocytes count in carregeenan-induced air pouch in rats compared to negative control group.

The values showed the percentage of inhibition of mean absolute leukocytes count.

Mean of leukocyte count for neutrophils and monocytes were calculated to obtain the percentage inhibition for both cells. Percentage of inhibition was calculated firstly by obtaining the difference between negative control with other group of administration. Then the values were divided with negative control and multiply 100 to obtain the percentage. The group treated with 1ml essential oil shows higher percentage inhibition of leukocytes count (69.76%) when compared with negative control group followed by 50mg/kg polypeptide-K compound (63.95%), 2ml essential oil (62.81%), 10mg/kg polypeptide-K compound (49.32%) and lastly positive control group (48.89%).

Group of Treatment	**Neutrophils & Monocytes (10^3µl)**	**Percentage of inhibition (%)**
Negative control (5ml/kg Distilled water)	54.907±10.667	-
Positive control (10mg/kg Indomethacin)	28.063±3.254	48.89
10mg/kg Polypeptide-K	27.826±4.281	49.32
50mg/kg Polypeptide-K	19.792±5.187	63.95
1ml per rat Essential oil	16.605±4.447	69.76
2ml per rat Essential oil	20.420±4.238	62.81

Table 4.5: Percentage inhibition leukocytes of polypeptide-K and essential oil on carrageenan-induced air pouch in rats

4.3 Determination of Interleukin 10 (IL-10) from Exudates Collection in Carrageenan-induced Air Pouch

The highest concentration of Interleukin 10 shown in 1ml essential oil group 483.377±84.818 followed by negative control group 402.623±80.701. There is no significantly difference ($p>0.05$) of IL-10 concentration between groups.

Group of Treatment	**Mean Concentration of Interleukin-10 (pg/ml)**
Negative control (5ml/kg Distilled water)	402.623±80.701
Positive control (10mg/kg Indomethacin)	240.667±23.212
10mg/kg Polypeptide-K	335.152±121.031
50mg/kg Polypeptide-K	253.942±65.733
1ml per rat Essential oil	483.377±84.818
2ml per rat Essential oil	338.848±33.520

Table 4.6: Effect of polypeptide-K and essential oil on mean concentration of interleukin 10 in carrageenan-induced air pouch

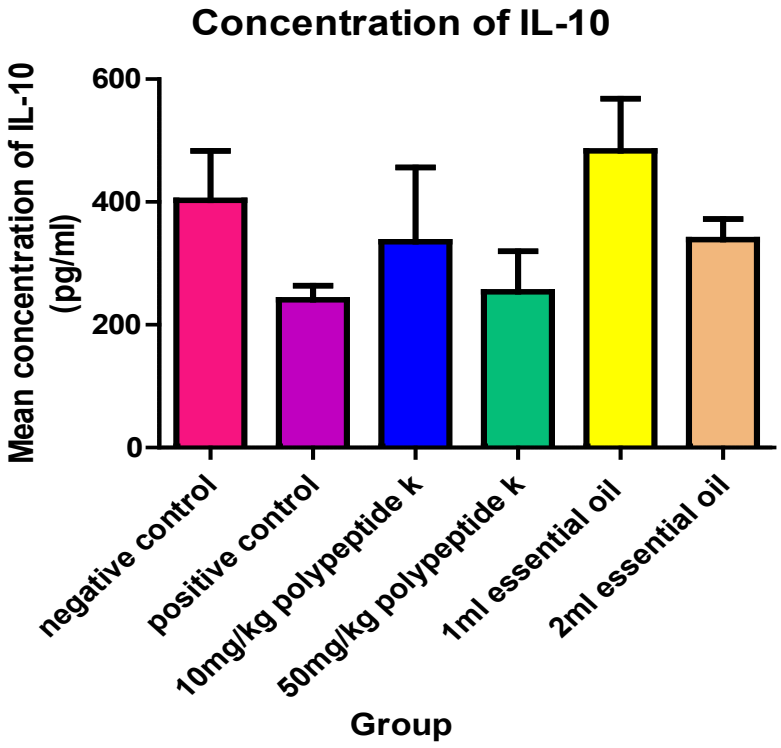


Figure 4.7: Effect of polypeptide-K and essential oil in carrageenan-induced air pouch in rats. The values showed mean±S.E.M concentration of IL-10.

+94.4 Determination of Nitrite from Exudates Collection in Carrageenan-induced Air Pouch

The highest concentration of nitrite was shown in negative control group 65.798±16.204. There is significantly difference ($p<0.05$) of nitrite concentration between group. There is significant decrease ($p<0.05$) of nitrite concentration in positive control group and 50mg/kg polypeptide-K compound.

Group of Treatment	**Mean Concentration of Nitrite (µmol/gram)**
Negative control (5ml/kg Distilled water)	^{a}65.798±16.204
Positive control (10mg/kg Indomethacin)	^{b}23.748±6.550
10mg/kg Polypeptide-K	^{a}28.530±11.174
50mg/kg Polypeptide-K	^{b}14.352±3.528
1ml per rat Essential oil	^{a}55.919±9.809
2ml per rat Essential oil	^{a}25.798±5.410

a-b Mean with different superscripts differ significantly ($p<0.05$)

Table 4.7: Effect of polypeptide-K and essential oil on mean concentration of nitrite in carrageenan-induced air pouch

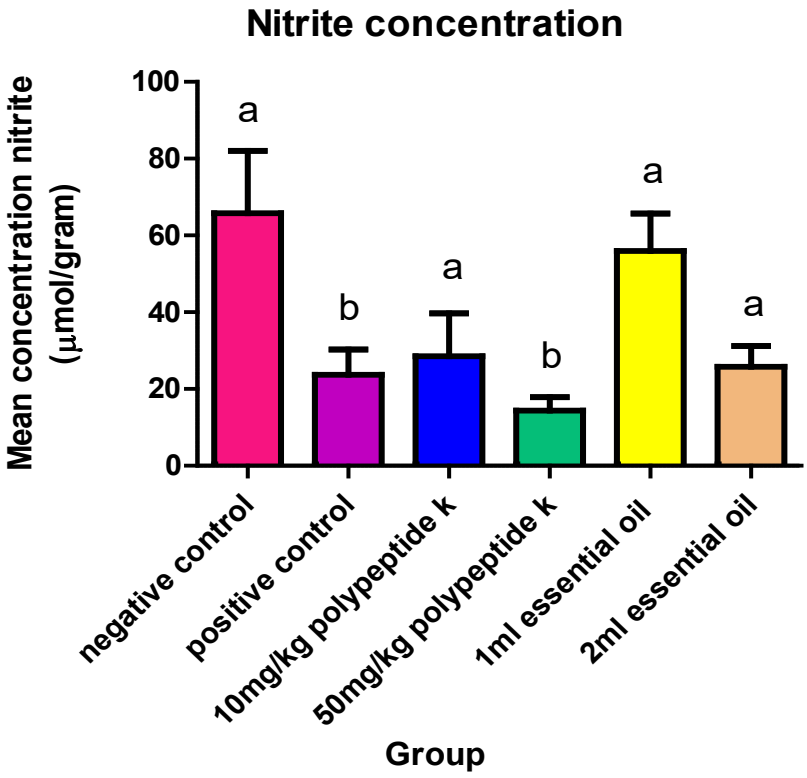


Figure 4.8: Effect of polypeptide-K and essential oil in carrageenan-induced air pouch. The values showed mean±S.E.M concentration of Nitrite.

a-b Mean with different superscripts differ significantly ($p<0.05$)

CHAPTER V

DISCUSSION

5.1 Carrageenan-induced Paw Edema

In carrageenan-induced paw edema, 0.1ml of 1% carrageenan was used to induce inflammation response on the right hind paw of rat. There is significant difference ($p<0.05$) between group of treatment on the reduction of paw edema volume. After 60 minutes carragenan injection, positive control group, 50mg/kg polypeptide-K compound, 1ml essential oil, and 2ml essential oil shown significantly decrease ($p<0.05$) paw edema volume. Oral administration of 10mg/kg and 50mg/kg polypeptide-K showed better effect in reducing the paw volume as compared to the Indomethacin which is the positive control. At 60 minutes after carrageenan injection, the group treated with 50mg/kg polypeptide K compound shows higher percentage inhibition of edema (74.75%), followed by 10mg/kg polypeptide-K compound (63.02%), 2ml essential oil administration (59.64%), 1ml essential oil administration (56.06%), and lastly positive control group (31.21%). The results showed that 50mg/kg polypeptide-K

compound was more potent than Indomethacin. 50mg/kg polypeptide-K may have potential in the suppression of fluid infiltration at the early phase of inflammation. It also indicates that this compound may affect membrane stabilization to reduce vascular permeability and inhibits various inflammatory mediators (Lee *et al*., 2009).

Carrageenan will cause a release of chemicals mediators such as bradykinin, serotonin and histamine at the early phase which is 0 to 1 hour, followed by release of prostaglandins and nitric oxide at late phase of inflammation which is 1to 6 hour (Giselle *et al*., 2007). The release of chemical mediators is induced by the cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) pathway (Nantel *et al*., 1999). The response of the carrageenan-induced inflammation was marked by the edema formation with increased in fluid infiltration (Olajide *et al*., 2000; Vinegar et al., 1969). Exudation of fluid and plasma proteins lead to a local edema formation consist of leukocyte-dependent and leukocyte-independent components (Lazzarani *et al*., 2006; Garcia-Lerne, 1989).

Based on the result obtained in this experiment, there is significant decrease ($p<0.05$) in paw volume after 60 minutes carrageenan injection. The highest percentage inhibition of paw volume at 60 minutes was in the 50mg/kg polypeptide-K group (74.75%). This evidence may due to anti-inflammatory action of 50mg/kg polypeptide-K related to the inhibition of one or more intracellular signaling pathways that involved in the release of several inflammatory mediators (Mendes *et al*., 2010).

5.2 Carrageenan-induced Air Pouch

In carrageenan-induced air pouch, sterile air were injected into the subcutaneous at the back of rats to form pouch that act as a reservoir for the cells infiltration and mediators that involves in inflammatory reaction (Nasrin *et al*., 2010). Inflammatory reactivity of the air pouch lining increased with time as the lining develops more organized surface layer of mononuclear phagocytes and fibroblastic cells that represent same as synovial cavity (Martin *et al*., 1994). After the injection of carrageenan into the pouches, there will be increase total leukocytes count and well as mediators that involves in inflammatory reaction (Shin *et al*., 2009). Cell recruitment during inflammation depends on the release of local mediators that will lead to vascular and tissue change such as cell migration, plasma exudation and production of mediators (Mendes *et al*., 2010). The recruitment of leukocytes is due to the release of the mediators.

Based on the result, there is significantly decrease ($p<0.05$) in total leukocytes count in 50mg/kg polypeptide-K group, 1ml essential oil and 2ml essential oil group. For the absolute leukocytes count, there is significantly decrease ($p<0.05$) of neutrophils count between group of treatment, meanwhile for the monocytes count, the significantly decrease shown in all group of treatment except for 10mg/kg polypeptide-K group. Essential oil may have potential in inhibition of the synthesis of many inflammatory mediators that involves in the cell migration. Other than that it also may be due to suppression of vascular leakage (Mendes *et al*., 2010). But the mechanism of action not

yet understood. The result shown that essential oil is more effective in reducing the number of leukocytes count compared to polypeptide-K and positive control group. This may indicate that the effect of essential oil was shown in the late phase of inflammation due the collection of exudates were taken 6 hours after injection of carrageenan.

Besides that, for the exudates volume, there is no significant different between group of treatment. In this inflammation model, the samples were collected 6 hour after carrageenan was induced. So 6 hour was categorized as late phase in acute inflammation. Air pouch model is not suitable for the fluid infiltration studies. This is because due to late samples collection, the fluid may not remain in the pouches. The fluid may enter the other circulation and retain the cells in the pouches.

5.3 Determination of IL-10 concentration

Interleukin 10 (IL-10) is one of the cytokine that responsible in suppressing the inflammatory response by inhibit the synthesis of proinflammatory cytokines. Where there are too many inflammatory cells in the system, IL-10 will inhibit the migration of inflammatory cells at the site of action (Yasunori *et al*., 1999). *Momordica charantia* juice had up regulated the secretion of IL-10 in in-vitro studies (Lin & Tang, 2008). Based on the result, there is no significant different of IL-10 concentration between group. The 1ml essential oil group showed the highest concentration of IL-10. It may indicate that 1ml essential oil exhibited anti-inflammatory activity by elevating anti-inflammatory IL-10 production and reducing the expression of iNOS and COX-2

pathway (Mueller *et al*., 2010). Meanwhile, for the other group of treatment, there is suppression of IL-10 concentration when compared to the negative control group. In the air pouch model, the samples were collected 6 hours after carrageenan-induced which at this hour it is categorized as late phase of acute inflammation. The induction of anti-inflammatory gene expression by IL-10 was initiated within minutes (Murray, 2005) where IL-10 is primarily secreted by activated macrophages and dendritic cells (Peter, 2006). Due to late samples collected the concentration of IL-10 had reduced.

5.4 Determination of Nitrite concentration

Nitric oxide synthesis by nitric oxide synthase (NOS) involved in physiological processes. There are three isoforms of NOS which is type I neuronal NOS (nNOS), type III endothelial NOS (eNOS) and type II inducible-type NOS (iNOS). In inflammatory response, there will be activation of iNOS pathway which leads to the release of nitric oxide that will increase vascular permeability. The activation of iNOS is from the specific stimulants in some cell such as cytokine to induce inflammatory reaction (Denlinger *et al*., 1996). The expression and activity of iNOS is the major therapeutic target to treat various inflammatory diseases (Lee *et al*., 2007). In the experiment it is difficult to measure the nitric oxide itself because it is rapidly metabolized into the stable end product which is nitrite and nitrate (Moshage *et al*., 1997). But usually there will be detection of nitrite as the indicator of nitric oxide level. Based on the result there is significantly decreased ($p<0.05$) of nitrite concentration in positive control group and 50mg/kg polypeptide-K group. 50mg/kg polypeptide-K may have a potential in

suppressed of nitrite production by inhibited the signaling pathways of the mediators. Other than that, it may have potential in decreased of endothelial permeability by suppress the concentration nitrite production in inflammatory reaction (Ricardo *et al*., 2005).

CHAPTER VI

CONCLUSION

The result obtained from this study showed that both polypeptide-K and essential oil have potential in inhibiting the carrageenan-induced acute inflammation.

In carrageenan-induced paw edema, there is significant reduction ($p<0.05$) of paw volume at 60 minutes after carrageenan injection. 50mg/kg polypeptide-K shows highest paw volume inhibition. This compound may have potential in suppressing vascular permeability at the early phase of acute inflammation.

Meanwhile in carrageenan-induced air pouch, there is significant decreased of total leukocytes by 50mg/kg polypeptide-K, 1ml essential oil and 2ml essential oil group. But essential oil group show higher inhibition percentage of leukocytes count. There is also suppression of nitrite and elevation of IL-10 concentration in essential oil group. It is suggest that essential oil give an effective effect at the late phase of acute inflammation by suppressing the infiltration of inflammatory cells.

CHAPTER VII

RECOMMENDATION

As polypeptide-K and essential oil showed having potential to work as an anti-inflammatory agents, it is recommended that the next study will more focus on the mechanism of action of the polypeptide-K and essential oil towards the release of mediators involves in inflammation.

Other than that, since polypeptide-K and essential oil has potential to suppress the production of nitrite, it is recommended that there will more concentration of polypeptide-K and essential oil can be used in the next study as only two concentrations respectively that had been tested.

Furthermore is to identify the active compound contain in essential oil extracted from *Momordica charantia* seeds. It is believes that by knowing the active compound, it will produce faster effect as the anti-inflammatory agents. The other properties of polypeptide-K and essential oil should be studied since these compounds are available and easy to consume.

REFERENCES

Alessandra Braca, Tiziana Siciliano, Manuela D'Arrigo, Maria Paola Germanò, 2008, Chemical composition and antimicrobial activity of Momordica charantia seed essential oil, *Fitoterapia*. 79: 123–125

Arazi, A.U.Neumann, 2010, Modeling immune complex-mediated autoimmune inflammation, *Journal of Theoretical Biology*. 267: 426-436.

Bang Yeon Hwang, Do Ik Lee, Yun-Bae Kim, 2010, Anti-allergic effects and mechanisms of action of the ethanolic extract of Angelica gigas in dinitrofluorobenzene-induced inflammation models, *Environmental Toxicology and Pharmacology*. 30: 127–133.

Basch, E.; Gabardi, S.; Ulbricht, C. Bitter melon (Momordica charantia): a review of efficacy and safety. Am. J. Health-Syst. Pharm. 2003, 60, 356–359.

Cheng Xiao Qi, 2007, Effect of *Bixa orellana* on Histamine-Induced Acute Inflammation in Rats, Dissertation for Final Year Project, Department of Biomedical Sciences, Faculty of Medical and Health Sciences, University Putra Malaysia.

Christopher J.Morris, Carrageenan-Induced Paw Edema in the Rat and Mouse, Methods in Molecular Biology, 1, volume 225, Inflammation Protols, II, pg 115-121.

Chun-Ching Shih, Cheng-Hsiu Lin, Wei-Li Lin, 2008, Effects of *Momordica charantia* on insulin resistance and visceral obesity in mice on high-fat diet, *Diabetes Research and Clinical Practise*. 81: 134-143.

Cotran; Kumar, Collins (1998). *Robbins Pathologic Basis of Disease*. Philadelphia: W.B Saunders Company. ISBN 0-7216-7335-X.

Csaba SzabÓ, 2000, Pathophysiological Roles of Nitric Oxide in Inflammation, *Principles of Pathobiology*, Chapter 52: 841-872. Beverly, Massachusetts: Inotek Corporation.

Do Yeon Lee, Byung Kil Choo, Taesook Yoon, Myeong Sook Cheon, HyeWon Lee, A. Yeong Lee, Ho Kyoung Kim, 2009, Anti-inflammatory effects of *Asparagus cochinchinensis* extract in acute and chronic cutaneous inflammation, *Journal of Ethnopharmacology*. 121: 28–34.

Giselle F.Passos, Elizabeth S.Fernandes, Fernanda M. da Cunha, juliano Ferrcira, Luiz F. Pianowski, Maria M. Campos, Joan B. Calixto, 2007, Anti-inflammatory and anti-allergic properties of the essential oil and active compounds from *Cordia verbenacea*, *Journal of Ethnopharmacology*. 2007, 110:323-333.

Georgewill OA, Georgewill UO, Nwankwoala RNP, 2010, Anti-inflammatory effects of Morninga oleifera lam extract in rats, *Asian Pacific Journal of Tropical Medicine*. 133-135.

Han Moshage, 1997, Nitric Oxide Determinations, *Clinical Chemistry*. 43(4): 553-556.

Jun-Yan Tao, Guo-Hua Zheng, Lei Zhao, Jian-GuoWu, Xiao-Yu Zhang, Shu-Ling Zhang, Zhi-Jun Huang, Fu-Liang Xiong, Chong-Ming Li, 2009, Anti-inflammatory effects of ethyl acetate fraction from *Melilotus suaveolens* Ledeb on LPS-stimulated RAW264.7 cells, *Journal of Ethnopharmacology*. 123: 97–105.

José Galberto M. Costa, Eidla M. M. Nascimento, Adriana R. Campos and Fabiola F. G. Rodrigues, 2010, Antibacterial activity of *Momordica charantia* (Curcubitaceae) extracts and fractions, *Journal of Basic and Clinical Pharmacy*. Vol-002 Issue-001.

J.K. Grover, S.P. Yadav, 2004, Pharmacological actions and potential uses of *Momordica charantia*: a review, *Journal of Ethnopharmacology*. 93: 123–132.

Kanna P, 2004. Protein/polypeptide-k obtained from *Momordica charantia*. United States Patent 6831162.

Khanna P, Jain SC, Panagariya A (1981). Hypoglycemic activity of polypeptide-p from a plant source. J. Nat. Prod., 44: 648-655.

Keiichi Omote, Koji Hazama, Tomoyuki Kawamata, Mikito Kawamata, Yoshito Nakayaka, Masaki Toriyabe, Akiyoshi Namiki, 2001, Peripheral nitric oxide in carrageenan-induced inflammation, *Brain Research*. 912: 171–175.

Lazzarini Ricardo, Paulo César Maiorka, Jun Liu, Vassilios Papadopoulos, João Palermo-Neto, 2006, Diazepam effects on carrageenan-induced inflammatory paw edema in rats: Role of nitric oxide, *Life Sciences*. 78: 3027–3034.

Lee Cheng Lok, M. D. Yong Yean Sirn, M. D. Zuraini Ahmad, Azhar Yaacob, Muhammad Nazrul Hakim, 2011, Effects of polypeptide-K supplemented soft bun on blood glucose level in healthy adults, *International Journal of Nutrition and Metabolism*. Vol. 3(1), pp. 7-10.

Lin Aanonsen and J. Richardson. "Characterization of carrageenan-induced inflammation in the mouse" *Society for Neuroscience.* 22 (1996): 880.

Loren Laine, 2003, Gastrointestinal Effects of NSAIDs and Coxibs, *Journal of Pain and Symptom Management*. Vol. 25(2): 32-40.

L. O. Okunrobo, C. O. Usifoh, P. F. Ching & M. Bariweni : Anti-inflammatory evaluation of methanol extract and aqueous fraction of the leaves of Anthocleista djalonensis A. Chev (Gentianaceae).. *The Internet Journal of Pharmacology.* 2009 Volume 7 Number 1.

Mu Hong Lee, Jeong Min Lee, Sung Hoon Jun, Seung Ha Lee, Nam Wook Kim, Jun Ho Lee, Na Young Ko, Se Hwan Mun, Bo Kyung Kim, Beong Ou Lim, Dong Kug Choi, Wahn Soo Choi, 2007, The anti-inflammatory effects of *Pyrolae herba* extract through the inhibition of the expression of inducible nitric oxide synthase (iNOS) and NO production, *Journal of Ethnopharmacology*. 112: 49–54.

Monika Mueller, Stefanie Hobiger, Alois Jungbauer, 2010, Anti-inflammatory activity of extracts from fruits, herbs and spices, *Food Chemistry*. 122: 987–996.

Nasrin Maleki-Dizaji, Tahereh Eteraf-Oskouei, Ashraf Fakhrjou, Seyyed Hadi Maljaie, Alireza Garjani, 2010, The effects of 5HT3 receptor antagonist granisetron on inflammatory parameters and angiogenesis in the air-pouch model of inflammation, *International Immunopharmacology*.

Peter J Murray, 2006, Understanding and exploiting the endogenous interleukin-10/STAT3-mediated anti-inflammatory response, *Current Opinion in Pharmacology*. 6:379–386.

Sharif M. Al-Reza, Jung In Yoon, Hyo Jung Kim, Jong Sang Kim, Sun Chui Kang, 2009, Anti-inflammatory activity of seed essential oil from *Zizyphus jujube*, *Food and Chemical Toxicology*. 48:639-643.

Shin Sunhee, Jeong Hee Jeon, Dongsun Park, Ja Young Jang, Seong Soo Joo, 2009, Anti-inflammatory Effects of Ethanol Extract of *Angelica gigas* in a Carrageenan-Air Pouch Inflammation Model, *Exp. Animal*. 58(4):431-436.

Stefania Marzocco, Rosanna Di Paola, Ivana Serraino, Raffaella Sorrentino, Rosaria Meli, Giuseppina Mattaceraso, Salvatore Cuzzocrea, Aldo Pinto, Giuseppina Autore, 2004, Effect of methylguanidine in carrageenan-induced acute inflammation in the rats, *European Journal of Pharmacology*. 484: 341– 350.

Shreedhara Gupta, Bikramjit Raychaudhuri, Shouvik Banerjee, Banasri Das, Sibabrata Mukhopadhaya, Salil C. Datta, 2010, Momordicatin purified from fruits of Momordica charantia is effective to act as a potent antileishmania agent, *Parasitology International*. 59: 192–197.

Shrinivas Sharma, K.S. Lakshmi, Arjun Patidar, Abhinav Chaudhary, Sanjay Dhaker, 2009, Studies on anti-inflammatory effect of aqueous extract of leaves of Holoptelea integrifolia, Planch. in rats, *Journal of Pharmacology*. Vol 41(2):87-88.

S.S.Mendes, R.R. Bomfim, H.C.R. Jesus, P.B Alves, A.F. Blank, 2010, Evaluation of the analgesic and anti-inflammatory effects of the essential oil of *Lippia gracilis* leaves, *Journal of Ethnopharmacology*. 129:391-397.

S. W. Martin, A. J. Stevens, B. S. Brennan, D. Davies, M. Rowland, and J. B. Houston, 1994, The Six-Day-Old Rat Air Pouch Model of Inflammation: Characterization of the Inflammatory Response to Carrageenan, *Journal of Pharmacological and Toxicological Methods*. 32(3): 139-147.

Valiollah Hajhashemi, Alieza Ghannadi, Sayed Karim Pezeshkian, 2002, Antinociceptive and Anti-inflammatory effects of Satureja hortensis L. extracts and essential oil, *Journal of Ethonopharmacology*. 82: 83-87.

Xiang L, Huang X, Chen L, Rao P, Ke L, 2007, The reparative effects of *Momordica charantia* Linn. extract on HIT-T15 pancreatic _ Cells. Asia Pac. *J. Clinical Nut*. 16(Suppl 1): 249-252.

Yasunori Sato, Tohru Ohshima, and Toshikazu Kondo, 1999, Regulatory Role of Endogenous Interleukin-10 in Cutaneous Inflammatory Response of Murine Wound Healing, *Biochemical and Biophysical Research Communications*. 265: 194–199.

Yu-Tang Tung, Meng-Thong Chua, Sheng-Yang Wang, Shang-Tzen Chang, 2008, Anti-inflammation activities of essential oil and its constituents from indigenous cinnamon (*Cinnamomum osmophloeum*) twigs, *Bioresource Technology*. 99: 3908–3913.

Yong, T., Mayhan, W.G., 1992. Effect of prostaglandin E1 on leukotriene C4-induced increases in vascular permeability of hamster cheek pouch. *Inflammation*. 16 (2), 159–167.

Yong, Y.K, 2007, Effect of Aqueous Extract *Bixa orellana* on Serotonin-Induced Inflammation, Dissertation for Final Year Project, Department of Biomedical Sciences, Faculty of Medical and Health Sciences, University Putra Malaysia.

APPENDIX A

Preparation of standard solution that had been used for calibration of the plethysmometer in the Carrageenan-induced paw edema model

Preparation of Normal Saline as the standard solution:

0.9g of Natrium Chloride dissolved in 100ml of distilled water to produce 0.9% of normal saline. 1g stick measurement was used as the reference calibration to make sure the solution level of normal saline is in the correct range.

Reference range for calibration 0.9g until 1.2g

APPENDIX B

Preparation of Carrageenan solution

To produce 1% of Carrageenan solution for paw edema model, 10mg of Carrageenan is dissolved in 1ml of normal saline and further being mixed using vortex mixer.

To produce 0.5% of Carrageenan solution for air pouch model, 25mg of Carrageenan is dissolved in 5ml of normal saline and further being mixed using vortex mixer.

APPENDIX C

Griess Reaction Assay for Nitric Oxide (NO)

Method in Griess Reaction Assay:

i) 50µl of supernatant was pipette in each well in duplicate

ii) 50µl of $NaNO_2$ (100µM-0µM) pipette to each well duplicate and in 2 fold to make a 1:2 dilution for the generation of standard curve.

iii) 50µl of distilled water as negative control.

iv) 50µl of Griess Reagent was added to each well.

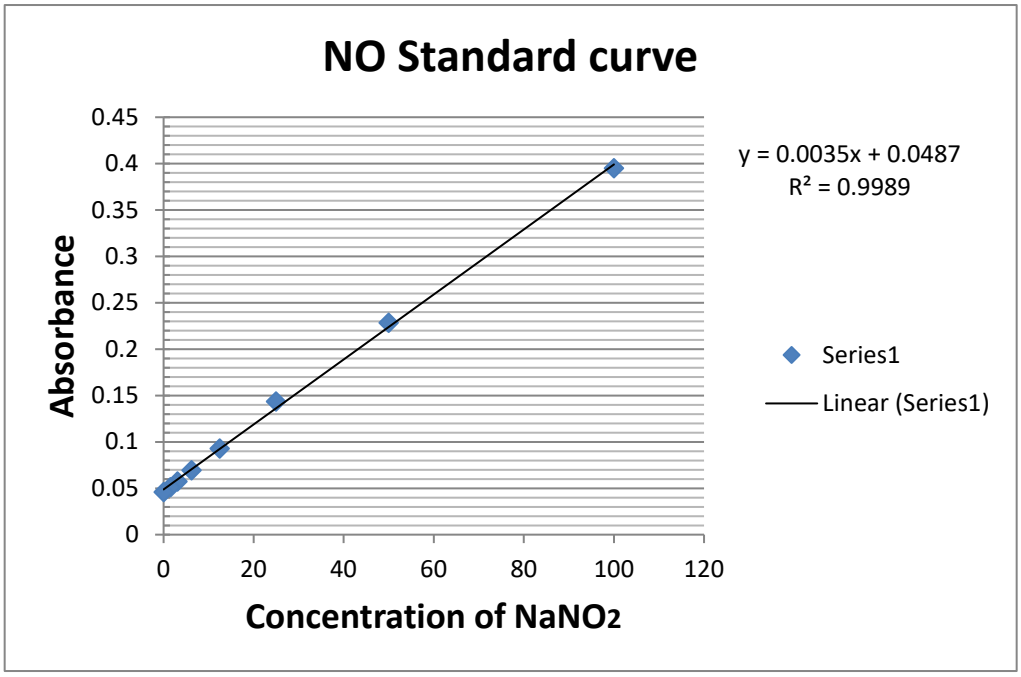
v) Absorbance of color change was read after 10 minutes incubation period at 548nm.

vi) Standard curve was generated and concentration of NO calculates using standard equation.

APPENDIX D

Standard curve for Nitric Oxide:

Concentration	0	1.5	3.1	6.2	12.5	25	50	100
Absorbance	0.0461	0.0513	0.0575	0.0694	0.0931	0.1436	0.2286	0.3951



APPENDIX E

Detection of Interleukin 10 (IL-10)

Using Rat IL-10 Platinum ELISA

i) The microwell strips was washed twice with 400µl Wash Buffer per well. The Wash Buffer was allowed to sit in the wells for about 10-15 seconds before aspiration.

ii) 100µl of Sample Diluent was added in duplicate to all standard wells. The sample wash prepared in 1:2 dilutions.

iii) 100µl of Sample Diluents was added in duplicate to the blank wells.

iv) 50µl of Sample Diluents was added to the sample wells.

v) 50µl of each sample was added in duplicate to the sample wells.

vi) 50µl of Biotin-Conjugate was added to all wells.

vii) The wells were covered with an adhesive film and incubate at room temperature for 2 hours.

viii) The microwell strips were then washed 3 times and 100µl of diluted Streptavidin –HRP was added to all wells including blank wells.

ix) Then incubated for another 1 hour.

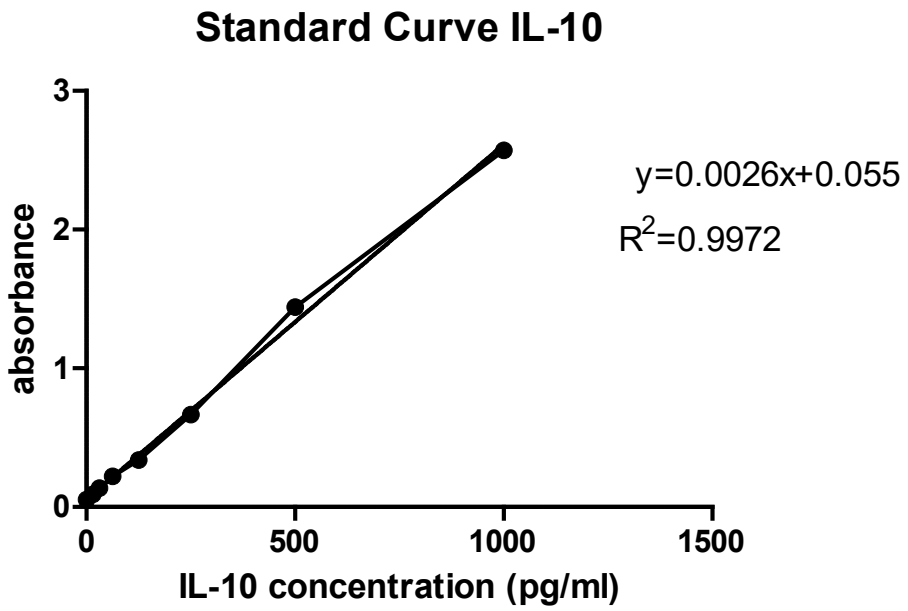
x) 100µl of TMB Substrate Solution was pipette to all wells and incubate at room temperature and avoid direct exposure to intense light.

xi) After incubation time, the reaction was stopped by pipetting 100µl of Stop Solution into each well.

xii) The absorbance was read using spectrophotometer at 450nm as the primary wavelength with 620nm as reference wavelength.

APPENDIX F

Standard Curve of Interleukin 10 (IL-10)



Concentration	0	15.6	31.3	62.5	125	250	500	1000
Absorbance	0.0536	0.0911	0.1359	0.2211	0.3381	0.6663	1.4408	2.5722

APPENDIX G

Differential leukocytes cell count

Negative control (5ml/kg distilled water)

cell/rat	1	2	3	4	5	6	AVERAGE
neutrophils	60	79	73	66	69	70	69.5
lymphocytes	17	5	7	11	8	6	9
monocytes	24	16	10	23	23	24	20
TOTAL	100	100	100	100	100	100	

Positive control (10mg/kg Indomethacin)

cell/rat	1	2	3	4	5	6	AVERAGE
neutrophils	60	59	55	55	67	63	59.83333
lymphocytes	25	28	29	29	22	24	26.16667
monocytes	15	13	16	16	11	13	14
TOTAL	100	100	100	100	100	100	

10mg/kg Polypeptide K compound

cell/rat	1	2	3	4	5	6	AVERAGE
neutrophils	69	47	58	57	57	79	61.16667
lymphocytes	17	30	23	14	23	7	19
monocytes	14	23	19	29	20	15	20
TOTAL	100	100	100	100	100	100	

50mg/kg Polypeptide K compound

cell/rat	1	2	3	4	5	6	AVERAGE
neutrophils	62	55	60	69	42	57	57.5
lymphocytes	24	17	11	11	21	17	16.83333
monocytes	14	28	29	20	37	26	25.66667
TOTAL	100	100	100	100	100	100	

50% essential oil (1ml per rat)

cell/rat	1	2	3	4	5	6	AVERAGE
neutrophils	68	61	56	57	59	60	60.16667
lymphocytes	19	18	16	22	23	25	20.5
monocytes	13	21	28	21	18	13	19
TOTAL	100	100	100	100	100	100	

100% essential oil (2ml per rat)

cell/rat	1	2	3	4	5	6	AVERAGE
neutrophils	44	43	50	63	65	64	54.83333
lymphocytes	36	25	30	22	17	18	24.66667
monocytes	20	32	20	15	18	18	20.5
TOTAL	100	100	100	100	100	100	