EVALUATION OF *Fructus Viticis* METHANOLIC CRUDE EXTRACT AS ANTIOXIDANT AND ANTI-INFLAMMATORY IN CARRAGEENAN INDUCED ACUTE PAW OEDEMA

NURUL HUSNA BINTI AZIZUL

UNIVERSITI SAINS MALAYSIA

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by

NURUL HUSNA BINTI AZIZUL

Dissertation submitted in partial fulfilment of the requirements for the Master of Health Sciences (Biomedicine)

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CERTIFICATE

This is to certify that the dissertation entitled 'Evaluation of *Fructus Viticis* Methanolic Crude Extract as Antioxidant and Anti-Inflammatory in Carrageenan Induced Acute Paw Oedema' is fine record of research work done by Nurul Husna binti Azizul during the period from February 2020 to September 2020 under my supervision. I have read this dissertation and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation to be submitted in partial fulfilment for the Master of Science (Biomedicine).

Supervisor,	Co-Supervisor,
.....	.....
Dr Wan Amir Nizam Wan Ahmad	Dr Suvik Assaw
Lecturer,	Post Doctoral Fellow,
School of Health Sciences,	School of Health Sciences,
Universiti Sains Malaysia,	Universiti Sains Malaysia,
Health Campus,	Health Campus,
16150 Kubang Kerian,	16150 Kubang Kerian,
Kelantan, Malaysia.	Kelantan, Malaysia.
Date:	Date: .....

DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

.....

(Nurul Husna Binti Azizul)

Date: .....

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Sincerely,

.....

(Nurul Husna binti Azizul)

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LIST OF SYMBOLS AND ABBREVIATION

α	Alpha
β	Beta
λ	Lambda
ι	Iota
ƙ	Kappa
µ	Micro
º	Degree
TNF	Tumor necrosis factor
DMSO	Dimethyl sulfoxide
LPS	Lipopolysachharides
NSAIDs	Non-steroidal anti-inflammatory drugs
NO	Nitric oxide
DAMPs	Damage-associated molecular patterns
IL	Interleukin
TGF	Tumor growth factor
NK	Natural killer
NETs	Neutrophils extracellular trap
M1	Inhibitory/ Killing macrophages
M2	Healing macrophages
NF-ƙB	nuclear factor kappa-light-chain-enhancer of activated B cells
HIF-1α	Hypoxia-inducible factors-1-alpha
STAT	Signal transducer and activator of transcription
i.p	Intraperitoneally
s.c	Subcutaneously
v/v	Volume/volume

w/v	Weight/Volume
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
PRRs	Pattern Recognition Receptors
PMNs	Polymorphonuclear Neutrophils
PMS	Pre-Menstrual Syndrome
TLR4	Toll-Like Receptor 4
DPX	Di-N-Butyl Phthalate in Xylene
H&E	Hematoxylin and Eosin
IFNγ	Interferon Gamma
LPS	Lipopolysaccharide
DCs	Dendritic cells
NK	Natural killer cells
ROS	Reactive oxygen species
LNMMA	NG-monomethyl-L-arginine

PENILAIAN EKSTRAK METANOL KASAR *Fruktus Viticis* SEBAGAI ANTI-OKSIDAN DAN ANTI-RADANG DALAM TAPAK TANGAN EDEMA AKUT YANG DIDORONG CARRAGEENAN

ABSTRAK

Keradangan merupakan mekanisma perlindungan semulajadi bagi melindungi badan daripada pelbagai kecederaan atau jangkitan dan akan menyebabkan rasa sakit. Tujuan kajian ini adalah untuk menilai kesan ekstrak buah tumbuhan pantai *Vitex rotundifolia* iaitu Fructus viticis sebagai agen anti-oksida dan anti-keradangan ke atas proses keradangan akut dalam edema tapak kaki oleh karagenan. Fructus viticis ialah buah kepada *Vitex rotundifolia*, dan telah digunakan secara tradisional bagi merawat keradangan. Sebanyak 30 ekor tikus Sprague-Dawley jantan (8-12 minggu dengan berat badan 220-280 g) telah digunakan dalam kajian ini. Semua tikus telah dibahagikan kepada kumpulan rawatan yang berbeza dimana setiap kumpulan mengandungi 6 ekor tikus iaitu Kumpulan: A (Vehicle-DMSO + Saline); B (Vehicle-DMSO + 2% λ-Carrageenan); C (Extract + Saline); D (Extract + 2% λ-Carrageenan); E (LNMMA + 2% λ-Carrageenan). Vehicle (DMSO) dan ekstrak disuntik dengan suntikan subplantar 30 minit sebelum induksi keradangan dan kesakitan akut menggunakan 100 uL 2% λ-carrageenan atau saline ke kaki kanan belakang tikus. Edema kaki diukur dengan menggunakan vernier caliper digital, tingkah laku sakit ditaksir menggunakan ujian Randall-Selitto dan tekanan darah sistolik diambil menggunakan kaedah “tail-cuff” pada selang waktu yang berlainan (0.5, 2, 4. 6, 8, 12 dan 24 jam). Pada akhir kajian, semua haiwan telah dikorbankan dan sampel darah dikumpulkan melalui tusukan jantung untuk analisis darah penuh. Ekstrak metanol

‘Fructus viticis’ menunjukkan kesan anti-keradangan dengan mengurangkan pembentukan edema kaki pada 4 hingga 6 jam dan seterusnya menghasilkan kesan analgesik pada sela masa tertentu. Tambahan pula, keupayaan Fructus viticis mengurangkan keradangan dan juga kesakitan boleh dikaitkan dengan keupayaan ekstrak mengurangkan kemasukan sel imun terutamanya monosit/makrofaj ke dalam tapak kaki belakang tikus. Selain itu, kesan anti-keradangan dan analgesik oleh ekstrak Fructus viticis mungkin adalah kesan secara langsung aktiviti antioksida oleh Fructus viticis. Kesimpulannya, buah *V. rotundifolia* berpotensi untuk berkembang menjadi ubat anti-keradangan dan analgesic yang baik di bidang farmaseutikal pada masa hadapan.

EVALUATION OF *Fructus Viticis* METHANOLIC CRUDE EXTRACT AS ANTI-OXIDANT AND ANTI-INFLAMMATORY IN CARRAGEENAN INDUCED ACUTE PAW OEDEMA

ABSTRACT

Inflammation is a natural defense mechanism against various harmful stimuli that results in pain sensation. The aim of this study was to evaluate the fruits of *Vitex rotundifolia* known as Fructus Viticis methanolic crude extract as antioxidant and anti-inflammatory agents in carrageenan-induced acute paw oedema. Fructus viticis has been used as a traditional medicine for the treatment of inflammation. This acute (24 hour) inflammation study involved 30 male Sprague Dawley rats (8-12 weeks and 220-280 g). Rats were equally divided into 5 groups: A (Vehicle-DMSO + Saline); B (Vehicle-DMSO + 2% λ-Carrageenan); C (Extract + Saline); D (Extract + 2% λ-Carrageenan); E (LNMMA + 2% λ-Carrageenan). Vehicle (DMSO) and extract were injected by sub plantar injection 30 minutes prior to induction of inflammatory and acute pain using 100uL of 2 % λ-carrageenan or saline into the right hind paw. The paw oedema was determined by using a digital vernier caliper, pain behaviour was assessed using Randall-Selitto test and the systolic blood pressure was taken using the tail cuff method at different time intervals (0.5, 2, 4. 6, 8, 12 and 24 hours). At the end of study, all animals were euthanized and blood sample were collected via direct cardiac puncture for full blood count analyses.. Methanolic extract of the Fructus viticis exhibits anti-inflammatory effects by delaying the development of carrageenan-induced paw oedema at 4 h to 6 h which subsequently produces analgesic effect at certain period. Moreover, the ability of Fructus viticis extract to reduce the paw oedema as well as pain is suggested to be

associated with the potential of the extract reduce the infiltration of inflammatory cells which specifically the monocytes/macrophages into the hind paw. Furthermore, anti-inflammatory and analgesic effects of Fructus viticis extract might be the direct consequences of antioxidant activity of Fructus viticis. Overall, the fruit of *V.rotundifolia* have a potential to be developed into a novel anti-inflammatory and analgesic drugs in pharmacological field in future.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Inflammation is a natural occurrence as body encounters various harmful stimuli including pathogens, damaged cells, toxic compounds, irradiation microbial and viral infections, exposure to allergens, autoimmune and chronic diseases, obesity, consumption of alcohol, tobacco use, and a high-calorie diet (Medzhitov, 2008; Takeuchi and Akira, 2010; Freire and Van Dyke, 2013; Chen *et al*., 2018). It is a natural defense mechanism against these harmful stimuli thus it is vital for health as it is involved in removing of the harmful materials thus initiating the healing process (Hussain *et al*., 2016).

Generally, there are five (5) cardinal signs to characterize inflammation which are pain (*dolor*), redness (*rubor*), warmth (*calor*), swelling (*tumor*), and loss of function (*functio laesa*) that results from local immune, vascular and inflammatory cell responses to infection or injury (Brune and Hinz, (2004); Libby (2007); Takeuchi and Akira (2010). Various mechanisms involves in initiating inflammatory response depends on its triggering factors. There are numerous signalling pathways that triggered inflammation, including toll-like receptor (TLR) signalling, NF-κB pathway and JAK-STAT pathway. Although different factors initiate different pathways, there are common mechanisms involved. Cell surface pattern receptors recognise harmful stimuli then followed by activation of inflammatory pathways which subsequently up-regulates the inflammatory markers and the recruitment of inflammatory cells (Libby 2007; Chen *et al*., 2018).

Inflammatory response initiates when host tissues is triggered by harmful materials thus results in vascular dilation, enhanced permeability of capillaries, increase blood flow and leukocyte recruitment to the infected site (Freire and Van Dyke 2013). The first leucocyte recruited to the site of infection is polymorphonuclear neutrophils that involve in phagocytotic and microbicidal action. Then, second line defence mechanism is initiated by infiltration of mononuclear cells, monocytes and macrophages into the inflammation site that will clear cellular debris and neutrophils through phagocytosis (Freire and Van Dyke 2013).

Macrophage activity triggers the releasing of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, IL-12, TNF-α, GM-CSF and anti-inflammatory cytokines such as TGF-β (Chen *et al*., 2018). Additionally, activated macrophage also produced high concentration of nitric oxide and reactive oxygen species that can damage cell structures such as carbohydrates, nucleic acids, lipids, and proteins and alter their functions (Birben *et al*., 2012; Yanagisawa *et al*., 2008). Although inflammation is necessary for removing noxious stimuli, non-resolving inflammation can cause in pathological lesion. Failure to return damaged tissue to homeostasis and delaying of apoptosis can results in chronic inflammation including arthritis, asthma, cancers, cardiovascular diseases and periodontal diseases (Freire and Van Dyke, 2013).

Pain is one of the cardinal sign of inflammation due the releasing of molecular mediators that sensitise nociceptor neurons (Pinho-ribeiro *et al*., (2017), Tissue injury during inflammation causes more pain sensation thus resulting in hypersensitivity or ‘hyperalgesia’. Moreover, common drugs that used to treat inflammation and pain such as non-steroidal anti-inflammatory drugs (NSAIDs) are known to produce side effect with chronic disorder (Ong *et al*., 2007). Although it is highly effective and most common drug prescribed, some patients may experience

severe side effects as NSAIDs are known for multiple adverse effects, including gastrointestinal bleeding, cardiovascular side effects, and NSAID induced nephrotoxicity (Brune 2007; Wongrakpanich *et al*., 2018). Realizing the side effects of NSAIDs in treating inflammation and diseases, many researchers nowadays are looking for more safer treatment with less side effect such us utilizing and developing drugs from natural resources such as medicinal plants and herbs (Ausman *et al*., 2010).

In inflammatory response, leukocytes and mast cells activity enhance the production and release of reactive oxygen species (ROS) at the damaged area. ROS is a free radical molecule that is highly reactive and unstable as it carries one or more unpaired electrons (Arulselvan *et al*., 2016). ROS become stable by attacking the closest stable molecule and taking its electron meanwhile the attacked molecule can become a free radical by losing its electron and start a chain reaction cascade causing damage to the living cell (Arulselvan *et al*., 2016). Under physiological conditions, a dynamic equilibrium exists between the production of reactive oxygen species (ROS) and endogenous antioxidant defense as ROS are neutralized by antioxidant defense mechanisms (Suriyaprom *et al*., 2019). Oxidative stress occurs when ROS levels exceed levels of antioxidants as ROS can induce severe oxidative damage to macromolecules that leads to cellular dysfunction (Papada and Kaliora; 2019).

Vitex rotundifolia belong to the plant family Verbenaceae (Lee *et al*., 2013). Its dried ripened fruit has been used as a traditional medicine and is widely used in Korea, China, Japan, Pacific Island and Australia for the treatment of asthma, night blindness inflammation, headache, migraine, chronic bronchitis, eye pain, and gastrointestinal infections (Lee *et al*., 2013; Rani and Sharma, 2013; Chaudhry *et al*., 2019). Moreover, this plant also known as ‘Beach Vitex’ is widely distributed in

sandy shores area and can be found throughout sandy beaches of tropics and sub-tropics. In Southern Thailand and Northeastern of Malaysia, locals prepare traditional dessert made from rice flour and it is an important ingredient in 'nasi kerabu'. The leaf extract is added to give color and flavour, the dessert is served with grated coconut and granulated sugar." (Chan *et al*., 2016).

Studies have found that *V. rotundifolia* exhibits various pharmacology activities such as anti-inflammatory, cytotoxic, anti-cancer, anti-microbial, anti-nociceptive and anti-hyperprolactinemia (Chaudhry *et al*., 2019; Lee *et al*., 2013). MeOH extract of the fruits of *V. rotundifolia*, also known as Fructus viticis showed inhibitory effects on the nitric oxide (NO) production (Lee et al., 2013). Various phytochemical constituent can be isolated from the fruits of *V. rotundifolia*, Fructus viticis such as iridoids, phenylpropanoids, diterpenes, flavonoids, and lignans provide a potential explanation for the use of *V. rotundifolia* as a natural remedy with lesser side effects (Lee *et al.,* 2013; Kim and Shim, 2019).

1.2 Problem Statement

Inflammation is a natural and frequent occurrence when body encounters noxious stimuli in order to protect body from harm thus maintain vital health. However, non-resolving inflammation will results in adverse effects where it can cause pathological lesion and tissue injuries. Failure to return tissue to homeostasis and delaying of apoptosis will results in chronic inflammation including arthritis, asthma, cancers, cardiovascular diseases and periodontal diseases. Inflammation also associated with pain sensation thus resulting in hypersensitivity or 'hyperalgesia' which can interfere everyday activities. Although NSAIDs are common drug used to reduce inflammation and give analgesic effects, patients may experience various side effects

of the drugs together with GI complications, including bleeding and perforation. *V. rotundifolia* is widely used in Korea, China, and Japan for the treatment of inflammation. However, the usage of *V. rotundifolia* in treating inflammation is still not fully utilised in Malaysia. Studies has found that MeOH extract of *V. rotundifolia* fruits, Fructus Viticis possess various phytochemical constituent such as diterpenes, flavonoids, and lignans thus provide a potential explanation for the use of *V. rotundifolia* as a natural remedies for inflammation treatment with lesser side effects (Lee *et al.,* 2013; Kim and Shim, 2019).

1.3 Objectives

1.3.1 General Objectives

To evaluate Fructus Viticis methanolic crude extract as anti-oxidant and anti-inflammatory in carrageenan-induced acute paw oedema.

1.3.2 Specific Objectives

Achievable objectives

i. To evaluate the anti-inflammatory and analgesic effects of Fructus viticis on carrageenan-induced paw oedema.
ii. To elucidate the effect of Fructus viticis methanolic crude extract on infiltration of immune cells via full blood count analysis.
iii. To determine the anti-oxidant activity of Fructus viticis methanolic crude extract.

Non-achievable objectives

i. To determine anti-inflammatory effect of Fructus viticis methanolic extract on carrageenan-induced nitric oxide production in paw tissues.

ii. To elucidate the effect of Fructus viticis methanolic crude extract on infiltration of immune cells via histopathological analysis.

1.4 Hypothesis

Fructus viticis methanolic crude extract will have high antioxidant activity. Moreover, Fructus viticis methanolic crude extract will reduce paw oedema which subsequently inhibit pain. The inhibition of inflammation and pain is associated with the ability of the crude extract to inhibit carrageenan-induced NO and infiltration of specific inflammatory cells such as neutrophils and monocytes.

1.5 Rationale of Study

This study will accentuate natural products which are potent and have lesser side effects in preventing inflammatory diseases. This study also could promote a new database on our nation resources.

CHAPTER 2

LITERATURE REVIEW

2.1 Inflammation

Immune system is built with complex innate and adaptive component that are capable of responding to changes, thus maintaining tissue homeostasis (Belkaid and Hand, 2014). Inflammation is a natural defense mechanism against various harmful stimuli, so it is vital for health because it involves removing noxious substances and initiating healing processes (Hussain *et al*., 2016). Immune systems respond to these harmful stimuli as it is essential to maintain homeostasis and remove these noxious substances (Glass *et al*., 2010).

Inflammatory process can be organized into a number of sequential steps (Figure 2.1) (Shaykhiev *et al*., 2007). Inflammation occur when triggered by mechanical injuries, infections, allergens, toxins, or noxious xenobiotics that disrupt homeostasis and need to be sensed to elicit a protective response that aims to ultimately restore homeostasis (Chovatiya and Medzhitov, 2014). Tissue damages of infections will leads to the release of molecular signals termed damage-associated molecular pattern molecules (DAMPs), pathogen-associated molecular pattern molecules (PAMPs, released by invading pathogens), or alarmins that will activate tissue resident cells thus promotes the release of pro-inflammatory mediators, including pro-inflammatory cytokines, chemokines, vasoactive amines, and lipid mediators (Villeneuve *et al*., 2018). The releasing of various mediators including cytokines and chemokine by tissue cells will contribute to the dynamic process of leukocytes subset recruitment (neutrophils, monocytes etc.) to the site of injury by increase local blood

flow and vascular permeability (Crasci *et al*., 2018). Neutrophils are the first recruited cells and once in the tissue they initiate inflammation and the clearance of pathogens by promoting recruitment of additional granulocytes and monocytes, and undergo degranulation responses, oxidative burst and NETosis (an evolutionary conserved cell death process distinctly separate to apoptosis and necrosis that trap pathogens) (Jones *et al*., 2016).

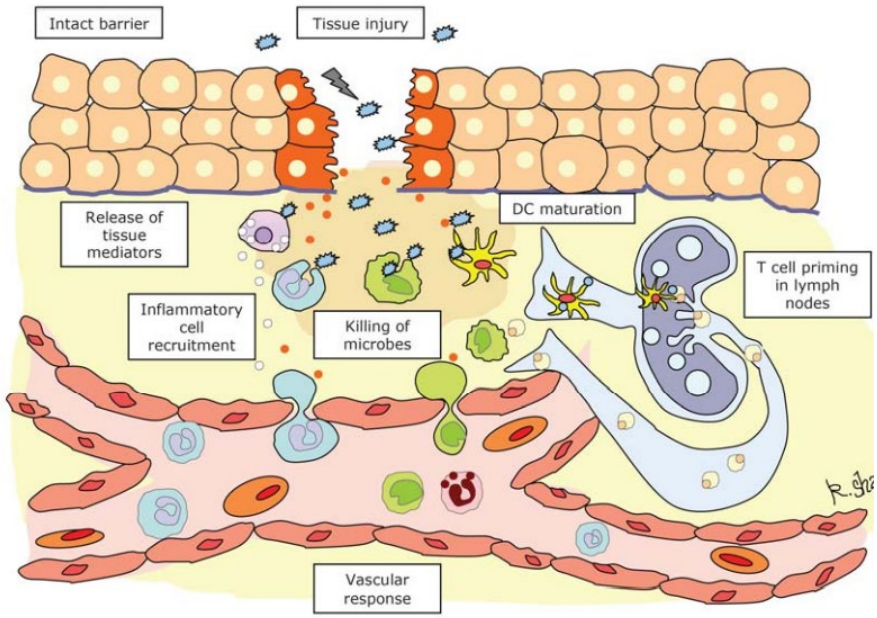

Figure 2.1 Inflammatory process. Inflammation is initiated by tissue injury, caused by physical damage to the tissue barrier or infection. Various mediators including chemokine, cytokines and vasoactive amines are released by epithelial cells and mast cells (violet) to increase vascular permeability and attract inflammatory cells from blood such as neutrophils (blue), monocytes (green) or eosinophils (pink), that migrate to the site of injury and kill microbes. Dendritic cells (yellow), matured in the presence of pathogens, migrate into regional lymph node, where they present antigen to T cells and thereby prime specific immune response (Shaykhiev et al., 2007).

Generally, classical signs of inflammation are redness (*rubor*), pain (*dolor*), heat (*calor*), swelling (*tumor*), and loss of function (*functio laesa*) (Ji *et al*., 2016). Heat (*calor*) sensation is caused by the of increased blood flow through dilated vessels and release of inflammatory mediators while oedema (*tumor*) is the result of exudation of fluid as well as cells being infiltrated to the site of infection. Moreover,

oedema and various mediators from the damaged and inflammatory response caused direct effect to the sensory nerves thus result in pain (*dolor*) sensation. Loss of function (*functio laesa*) can be caused by pain sensation and oedema thus interfere the movement of joint as well as replacement of functional cells into the scars tissue can result in loss of function (Gurenlian, 2009).

Inflammation is induced by loss of homeostasis, but also intentionally disrupts incompatible homeostatic processes and the resolution phase that restores homeostasis after inflammation indicates successful inflammatory response (Kotas and Medzhitov, 2015). Resolution of inflammation is important to avoid unnecessary tissue damage, reduction of energy, cellular and homoeostatic costs associated with inflammation and tissue damage, pain relief, remodelling, regeneration, and restoration of function (Gallo *et al*., 2017). Inflammation resolution involves neutrophil apoptosis and their phagocytic removal via efferocytosis, clearance of pro-inflammatory dead cells and cytokines, and recruitment or phenotype switching of macrophages to anti-inflammatory phenotype (Kulkarni *et al*., 2016). If inflammatory processes are not resolved, and active inflammation continues in a dysregulated fashion, it will lead to prolonged and chronic inflammation that usually associated with various chronic diseases such as arthritis, lupus, and periodontis (Zhou *et al*., 2016).

2.1.1 Acute Inflammation

Acute inflammation is a short-term process that require external stimulus as they response towards various harmful stimuli, usually occurring within minutes or hours and persist for a couple of days or weeks (Fritsch *et al*., 2019; Abudukelimu *et al*., 2018). It is activated by pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) through the Toll-like receptor (TLR)

systems and other innate immune receptors that able to recognize various harmful stimuli including viruses, bacteria, endogenous or exogenous danger signals, or foreign particles (MacLeod and Mansbridge, 2016).

Acute inflammation is characterized with specific cellular events, including increased permeability of the endothelium and epithelium, infiltration of polymorphonuclear leukocytes, inflammatory macrophages, and lymphocytes to sites of infection or injury, and subsequent tissue oedema (Duvall and Levy, 2016). The activation of transcription factors such as NF-κB and STAT3, inflammatory enzymes such as cyclooxygenase-2 (COX-2), matrix metalloproteinase-9 (MMP-9), and inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukins (IL) such as IL-1, IL-6, IL-8, and chemokines are the main molecular mediators of inflammatory response (Kunnumakkara *et al*., 2018). As inflammation achieves its goals and the wound is cleared of contamination, the acute wound healing process and inflammatory stage move to a reparative stage (MacLeod and Mansbridge, 2016).

2.1.2 Chronic Inflammation

Although inflammation inhibits infections or harmful materials from spreading across infection sites (Nathan and Ding, 2010), inflammation has been associated with various diseases such as rheumatoid arthritis, asthma, inflammatory bowel disease, neurodegenerative diseases and cancer. These diseases are caused by untreated or poor management of acute inflammation that leads to chronic inflammation (Hussein et al., 2013). Prolonged infiltration of various immune cells may turn acute inflammation into chronic inflammation, which persists over months or years, beyond the presence of the external stimuli (Abudukelimu *et al*., 2018).

"Chronic inflammation is defined as persistence of inflammatory processes beyond their physiological function, resulting in tissue destruction" (Nasef et al., 2017). Chronic inflammation are the results of altered mechanisms and the magnitude of acute inflammatory responses, potentially exacerbating and prolonging tissue inflammation and adversely affecting healing (Mu et al., 2016). Chronic inflammation may result in long-term tissue damage that caused by hypoxia, cell death, cellular necrosis, or autophagy, arthritis, and other autoimmune disorders, or from other non-acute injuries that also result in the recruitment of phagocytic and immune cells and in the production of pro-inflammatory cytokines (Ross, 2017). It results in increase of toxic products of inflammation, such as reactive oxygen species (ROS) and cathepsins released from lysosomes, which rupture in the process of cell death (Cox *et al.,* 2020). Chronic inflammation is distinguished by mononuclear cell infiltration such as monocyte and lymphocytes, fibroblasts proliferation, collagen fibers, and connective tissue formation, which ultimately result in formation of granuloma (Abdulkhaleq *et al*., 2018).

2.2 Role of immune cell during inflammation

The inflammatory response can be mediated by two types of immune system which are innate and adaptive immunity (Kinsey *et al*, 2008). Innate immune system is the first line host defense that responded to foreign materials before the adaptive immune system was able to take over (Whyte, 2007). The innate immune system is activated at the early stage of infectious or inflammatory states in a non-antigen-specific fashion and is comprised of immune cells such as neutrophils, monocytes/macrophages, dendritic cells (DCs), natural killer (NK) cells and natural killer T (NKT) cells. Meanwhile, the adaptive immune system reacts to specific antigens such as pathogens or dead self-cells after several days of infection,

including DC maturation and antigen presentation, CD4 and CD8 T lymphocyte proliferation and activation, and T to B lymphocyte interactions (Kinsey *et al.,*, 2008).

2.2.1 Neutrophils

Neutrophils is one of the polymorphonuclear leukocytes (PMNs), a special family of white blood cells are the most abundant leukocyte population in the blood, comprising 50–60% of the circulating leukocytes (25×10^9 cells) and one of the important components of the innate immune response (Fournier and Parkos, 2012). According to Selders *et al*., (2017), neutrophils are the first immune cells to response to injuries or pathogen infection and the presence of neutrophils indicates that these cell types play a critical role in the onset of inflammation. When body is injured of infected, neutrophils migrates out of the circulatory system through dilated vessels and recruited via chemotaxis to the site of infection (Selders *et al*., 2017).

Neutrophils are activated after exposure to numerous triggering factors including pro-inflammatory cytokines such as interferon (IFN)-γ and granulocyte/macrophage-colony stimulating factor (GM-CSF), which induces the activation of STAT transcription factor members, whereas tumor necrosis factor (TNF-α) and interleukin (IL-1β) induces the NF-κB classical inflammatory pathway (Kobayashi and DeLeo, 2009). Moreover, in the host immune system, neutrophils are essential as they can protect the host from rapidly dividing bacteria, yeast and fungal infections, possessing microbicidal mechanisms while producing reactive oxygen and nitrogen species, releasing proteolytic enzymes and microbicidal peptides from cytoplasmic granules (Shaw *et al*., 2010). In addition, neutrophils capable of destroying foreign antigens or pathogens by producing ROS and lytic enzymes while

releasing many chemokines recruiting additional neutrophils at the infection site (Selders *et al*., 2017).

Neutrophils phagocytic activity triggers the release of various cytokines and chemokine, including IL-1α, IL-1β, IL-1ε, IL-1RN, IL-6, IL-8, IL-10, IL-12β, IL-15, IL-18, CCL2 (MIP1α), CCL3 (MIP1β), CXCL1 (GROα), CXCL2 (MIP2α), CXCL3 (MIP2β), CXCL12 (SDF1), CCL20 (MIP3α), tumor necrosis factor (TNF)-α, vascular endothelial cell growth factor, and oncostatin M (Kobayashi *et al*., 2005). Massive neutrophil influx will lead to the formation of oedema and hemorrhage (Li *et al*., 2016). Study by Suo *et al*., (2014) revealed that the reduced neutrophil numbers in the inflamed tissue has led to a dramatic reduction of oedema formation. In addition, the migrating neutrophils participate in the cascade of events leading to mechanical hypernociception, by mediating the release of hyperalgesic molecules (such as MPO, MMPs, hypochlorite, superoxide anion, and PGE2) capable of activating nociceptive neurons and causing pain during inflammatory process (Rosas *et al*., 2017).

The number of the infiltrated neutrophils peaks in 6-24 hours after injury and declines rapidly 72-96 hours after injury (Yang and Hu, 2018). Due to present of antimicrobial and pro-inflammatory mechanisms, neutrophils need to return back to homeostatic state to avoid unnecessary tissue damage thus neutrophils clearance occur through apoptosis and senescent through a negative-feedback loop involving a cascade of cytokines, namely the IL-23–IL-17 G-CSF axis (Hajishengallis and Hajishengallis, 2014). Neutrophils apoptosis can be intrinsic (myeloid cell leukaemia-1 (Mcl-1)) or extrinsic (FasL, TRAIL and TNF-α) via activation of caspase-8 (Wright *et al*., 2010). In addition, neutrophils also often phagocytosed or

inhibited by macrophages or lymphocytes after digestion of pathogens to minimize the tissue damage (Liu *et al*., 2018).

2.2.2 Monocytes

Monocytes are second line defense of innate immune system where they migrate to sites of inflammation after neutrophil infiltration and can be sustained for days (Ingersoll *et al*., 2011). Monocytes are bone marrow-derived myeloid cells that belong to the mononuclear phagocytic system (MPS), a specialized system of phagocytic cells localized throughout the body (Lauvau *et al*., 2014). Neutrophils are capable of inducing the recruitment of other immune cells including monocytes by regulating the release of chemo attracting factors, such as cathepsin G and azurocidin, and neutrophils also can alter vascular permeability by inducing changes in the cytoskeletal structure of endothelial cells, thus promoting the transmigration of monocytes (Kumar *et al*., 2018).

Following conditioning by local growth factors, pro inflammatory cytokines and microbial products, monocytes escape apoptosis by differentiating into macrophages and dendritic cells, cells with a longer life span and can be found in almost every single organ (Parihar *et al.,* 2010). During homeostasis and inflammation, circulating monocytes leave the bloodstream and migrate into tissues in response to natural killer (NK) cell-produced interferon (IFN-γ) and chemokine receptor CCR2 and its ligands CCL2 and CCL7, then will further differentiate into macrophage or dendritic cell population (Shi and Pamer, 2011; Sprangers *et al*., 2016). Macrophages start to present at the site of infection at 24 hours after injury and the number of macrophages increases significantly 2 days after injury along with the rapid decline of the number of neutrophils (Yang and Hu, 2018).

Macrophages are a heterogeneous population of innate myeloid cells involved in health and disease (Xuan *et al*., 2015). They are scavenger cells that phagocytized cellular debris, invading microorganisms, neutrophils, and other apoptotic cells (Wynn and Vannella, 2016). According to Davies *et al*., (2013), macrophages have been functionally grouped into two classes: the 'M1-M2 paradigm'. M1 or classical activated macrophages (CAM) homing of pro-inflammatory (M1) and M2 or alternative activated macrophages (AAM) involves in anti-inflammatory that plays a different role in the process of inflammation (Xuan *et al*., 2015).

Pro-inflammatory monocytes in mice is characterized by $Gr1^{+}/Ly6C^{high}CCR2^{+}CX3CR1^{low}$ can differentiate into inflammatory macrophages and dendritic cells, while anti-inflammatory monocytes ($Gr1^{-}$/ $Ly6C^{low}CCR2^{-}$ $CX3CR1^{high}$) perform patrolling functions and differentiate to M2 macrophages (Orekhov *et al*., 2019). In human there are three monocyte subsets: classical ($CD14^{high}/CD16^{-}$), intermediate ($CD14^{high}/CD16^{+}$), and non-classical ($CD14^{low}/CD16^{+}$) where non-classical monocytes are the most pro-inflammatory in response to TLR stimulation (Ong *et al*., 2018; Gjelstrup *et al*., 2018). Monocytes that are circulating in the blood stream are short-lived and undergo spontaneous apoptosis under normal condition (Parihar et al., 2010). Classical monocytes have a very short circulating lifespan (mean 1.0 ± 0.26 d) whereas intermediate monocytes have a longer lifespan (mean 4.3 ± 0.36 d) and non-classical monocytes have the longest lifespan in blood (mean 7.4 ± 0.53 d), before either leaving the circulation or dying (Patel *et al*., 2017).

2.2.2 (a) Inflammatory Macrophages (M1)

CAM or M1 macrophages (Figure 2.2) can be activated by lipopolysaccharide (LPS) upon interaction with toll like receptors (TLRs) and IFN

signaling (Liu *et al*., 2014). On the other hand, carrageenan can induced TLR activation as carrageenan can activate both TLR2 and TLR4 and mediate NF-κβ pathway, similar to LPS (Shalini *et al*., 2015) M1 macrophages are characterized by enhanced expression of MHC class II and high production of pro-infammatory cytokines (Haloul *et al*., 2019). In addition, M1 macrophages also can be activated by IFN-γ and TNF-α (Yao *et al*., 2019).

When the pathogen associated molecular patterns (PAMPs) presented in bacteria are recognized by pathogen recognition receptors (TLRs), macrophages are activated and producing a large amount of pro-inflammatory mediators like cytokines IL-1β, IL-6, IL-12, IL-18 and IL-23, TNF-α, and type I IFN; and several chemokines such as CXCL1, CXCL3, CXCL5, CXCL8, CXCL9, CXCL10, CXCL11,CXCL13, and CXCL16; CCL2, CCL3, CCL4, CCL5, CCL8, CCL15, CCL11, CCL19, and CCL20; as well as CX3CL1; which induce Th1 response activation, facilitate complement-mediated phagocytosis, (Lu *et al*., 2018; Atri *et al*., 2018). M1 macrophages also can induce inducible NO synthase (iNOS), the enzyme that produces large amounts of NO that is not only cytotoxic, but produces toxic metabolites that establish M1 killing machinery and type I inflammation (Ley, 2017). It is found that NF-κB and STAT1 are the two major pathways involved in M1 macrophage polarization and result in microbicidal and tumouricidal functions (Yao *et al*., 2019).

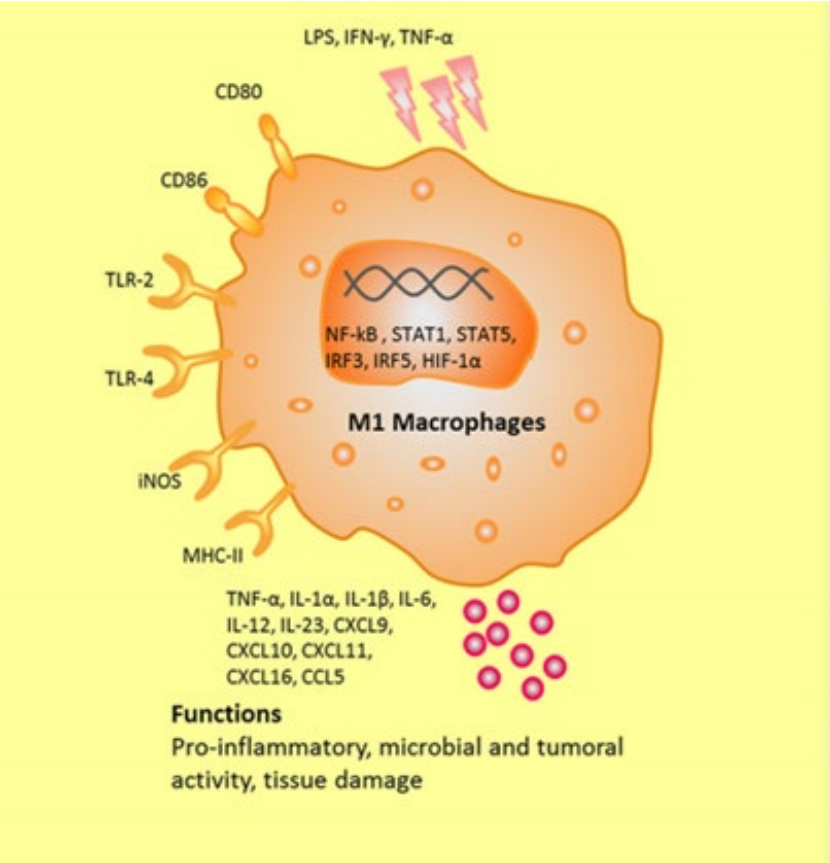

Figure 2.1 Classical activated macrophages (CAM) (Yao *et al*., 2019)

2.2.2 (b) Inflammatory Macrophages (M2)

M2 macrophages (Figure 2.3) are the result of the stimulation of Th2 signature cytokine interleukin-4 (IL-4) or IL-13 (Yu *et al*., 2019). M2 macrophage polarization can be induced by downstream signals of cytokines IL-4, IL-13, IL-10, IL-33, TGF-β, and they also can be activated by up-regulation of cytokines and chemokine, such as IL-10, TGF-β, CCL1, CCL17, CCL18, CCL22, and CCL24 (Yao *et al*., 2019). Macrophage M2 polarization involves tyrosine phosphorylation and activation of a signal transducer and activator of transcription 6 (Stat6), which mediates the transcriptional activation of M2 macrophage-specific genes such as arginase 1 (Arg1), mannose receptor 1 (Mrc1), resistin-like α (Retnla, Fizz1),

chitinase-like protein 3 (Chil3, Ym1), and the chemokine genes CCL17 and CCL24 (Yu *et al*., 2019).

M2 macrophages secrete anti-inflammatory cytokines like IL-10, CCL18 and CCL22 (Genin *et al*., 2015). In addition, M2 macrophages can produce IL-4 and IL-13 which could induce M2 polarization in while IL-10 can affect the morphology of IL-4 and IL-13 on macrophages, can downregulate the expression of MHC class II molecules, and has variable influences on mannose receptor expression, leading to decreased fluid-phase and mannose receptor-mediated endocytosis while TGF-β could uniquely inhibit inflammation through reducing iNOS-specific activity and decreasing iNOS protein production (Bi *et al*., 2019). Therefore, M2 macrophages is important in clearing the apoptotic cells, alleviation of inflammatory responses, and promotion of wound healing (Suzuki *et al*., 2017).

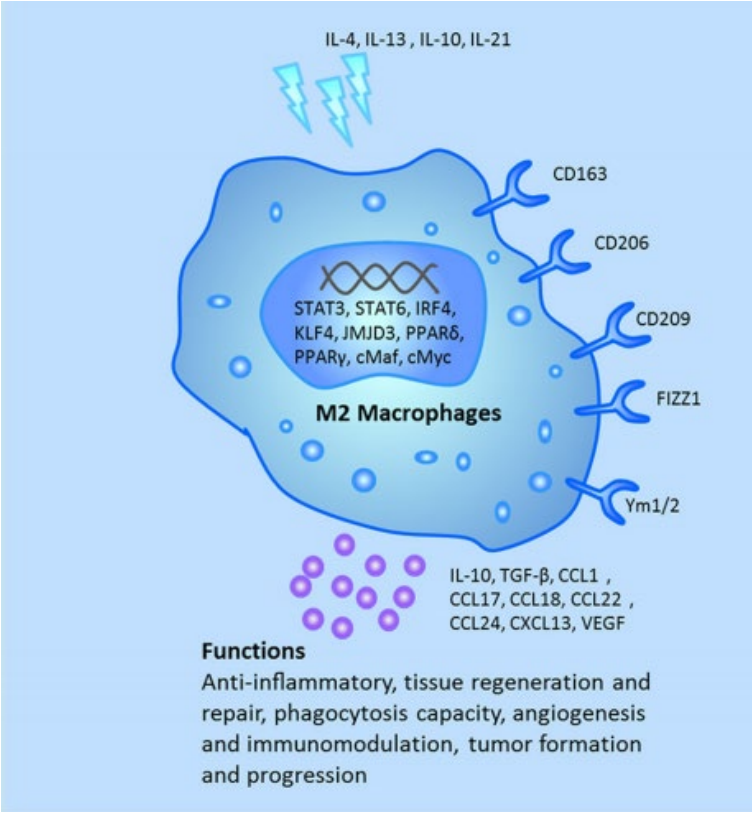

Figure 2.2 Alternative activated macrophages (AAM) (Yao *et al*., 2019)

2.3 NF- κB signaling in inflammation

NF-κB plays an important role as a mediator of the effects of inflammation and oxidative stress upon immune function (Arranz *et al*., 2010). NF-κB transcription factor involves in the inflammatory response by regulating the expression of various genes encoding pro-inflammatory mediators such as cytokines, chemokine, growth factors and inducible enzymes (Hussein *et al*., 2013). NF-κB has been associated in the pathogenesis of a number of inflammatory diseases, such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), multiple sclerosis, atherosclerosis systemic lupus erythematosus, type I diabetes, chronic obstructive pulmonary disease and asthma (Liu *et al*., 2017; Arranz et al., 2010). In addition, NF-κB present in the cytoplasm and is consists of five structurally related members, including NF-κB1 also known as p50, NF-κB2 also named p52, RelA also named p65, RelB and c-Rel, which mediates transcription of target genes by binding to a specific DNA element, κB enhancer, as various hetero- or homo-dimers (Liu *et al*., 2017).

Generally, NF-κB present as an active heterotrimer consisting of p50, p65 and IκBαsubunits (Brodsky *et al.,* 2010). p65 and p50 exist normally in the cytoplasm as an inactive complex by binding to inhibitory factor, IκBα, thereby blocking NF-κB nuclear translocation. Upon stimulation with inflammatory stimuli, IκBα is phosphorylated by IκB kinase (IKK) and separated from the NF-κB subunits which lead to its degradation. The free NF-κB is translocated into the nucleus and acts as transcription factor. In the nucleus, NF-κB dimers combine with target DNA elements to activate transcription of genes encoding for proteins involved in inflammation. In inflammation, activated NF-κB regulates transcription of IL-1b, IL-6, iNOS, COX-2 and TNF-α (Hussein et al., 2013).

There are various stimuli that able to initiate NF-κB pathway, including ligands of various cytokine receptors, pattern recognition receptors (PRRs), TNF receptor (TNFR) superfamily members, as well as T-cell receptor (TCR) and B-cell receptor (Liu *et al*., 2017). Toll-like receptors (TLRs) are transmembrane receptors that able to recognize the triggering factors such as bacteria lipopolysaccharide (LPS) thus activate the innate immune system. Figure 2.4 showing binding of lipopolysaccharide (LPS) to toll-like receptor 4 (TLR4) that is primarily expressed in macrophages initiates several signal transduction pathways, including NF-κB (Shih *et al*., 2018). Similar with LPS, carrageenan up regulated the mRNA and protein level expressions of both TLR2 and TLR4, thus activation of TLR-NF-κB signalling in carrageenan also can induce inflammation (Shalini *et al*., 2015).

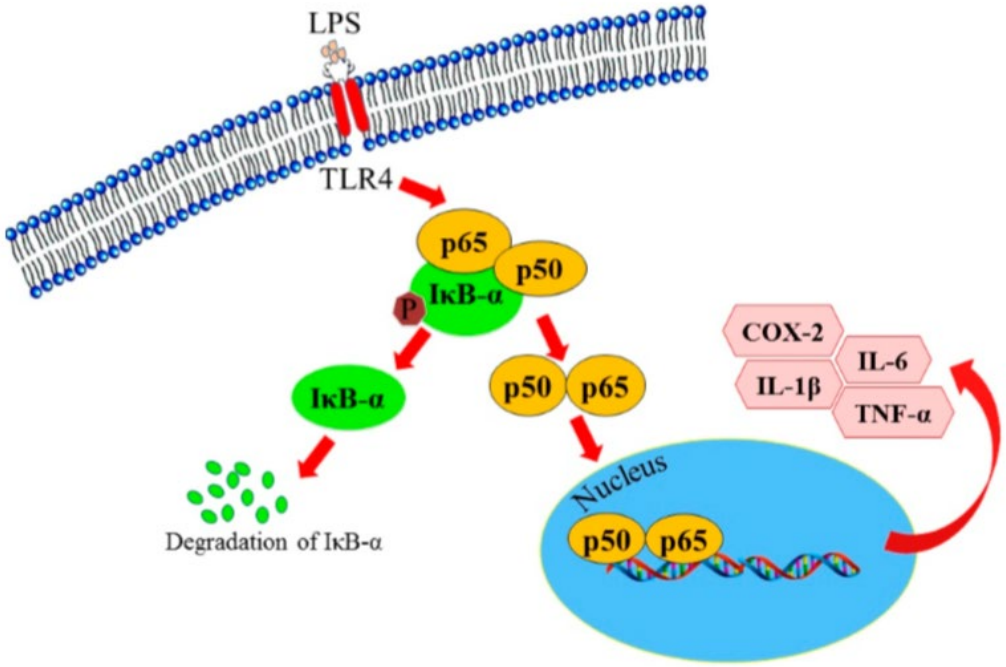

Figure 2.3 TLR4-mediated NF-κB signaling pathway (Shih *et al.,* 2018).

2.4 Inflammatory Pain

Nociceptors are receptors on nociceptive primary sensory neurons in the peripheral nervous system (Ji *et al*., 2016). It innervates peripheral tissues including the skin, respiratory, and gastrointestinal tracts, which are often exposed to numerous harmful stimuli including pathogens. Nociceptors sensory neurons are specialized to detect potentially damaging stimuli, protecting the host body by initiating the sensation of pain and eliciting defensive behaviors (Chiu *et al*., 2013). According to Omoigui, (2007), pain is currently defined by the International Association for the Study of Pain (IASP) as 'an unpleasant sensory or emotional experience associated with actual or potential tissue damage, or described in terms of such damage'. Inflammatory responses in the peripheral and central nervous systems have been associated with the development and persistence of many pathological pain states (Zhang and An, 2007). Pain serves obvious physiological functions, such as warning of potentially dangerous stimuli or drawing attention to inflamed tissue (White *et al*., 2005).

Pain can cause hyperalgesia, allodynia and spontaneous pain (Stemkowski and Smith, 2012). Hyperalgesia is a condition where the sensitivity to pain is increased abnormally, resulting in hypersensitivity due to the sensitised nociceptive nerve endings. On the other hand, allodynia is a condition when body experience pain from a stimuli that normally do not cause pain. For example touch, light pressure, or moderate cold or warmth can cause pain when applied to apparently normal skin. Spontaneous pain is the consequence of chronic pain thus resulting in non-evoke pain sensation (Stemkowski and Smith, 2012).

Inflammation are often been associated with pain due to the production of mediators such as pro inflammatory cytokines, chemokines, PGE_2, and NO mainly

by microglial cells and by other non-neuronal cells of the nervous as well as immune cells such as macrophages, thus contribute to pain hypersensitivity by activating nociceptive neurons in the CNS and in the peripheral nervous system (PNS) (Carniglia *et al*., 2016). Studies by Cui *et al*., (2000) shown that there was a highly significant difference in the number of monocytes/macrophages, IL-6 and TNF-α positive cells between allodynic and non-allodynic rats, suggesting that these inflammatory components are associated with the development pain. Furthermore, macrophages can induce nerves growth factors (NGF) via production of cytokines such as TNF-α, IL-6 and IL-1β thus contributes to the generation of neuropathic pain.

According to Zhang and An, (2007), there are abundant of evidence that associate pro-inflammatory cytokine produced by activated macrophages with the process of pathological pain. For example, IL-1β was found to increase the production of substance P and prostaglandin E2 (PGE_2) in a number of neuronal and glial cells thus results in hyperalgesia. IL-6 involved in microglial and astrocytic activation as well as in regulation of neuronal neuropeptides expression thus contributes to the development of neuropathic pain behavior following a peripheral nerve injury. Another cytokine, TNF-α acts on several different signaling pathways through two cell surface receptors, TNFR1 and TNFR2 to regulate apoptotic pathways, NF-κβ activation of inflammation, and activate stress-activated protein kinases (SAPKs) also shown to play important roles in both inflammatory and neuropathic hyperalgesia.

2.5 Non-Steroidal Anti-inflammatory Drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most widely prescribed and common drugs used in the world (Burian and Geisslinger, 2005). NSAIDs are frequently used for the treatment of signs and symptoms of inflammation as well as an antipyretics, analgesics and inhibitors of platelet aggregation (Ulrich *et al*., 2006). The anti-inflammatory action of non-steroidal anti-inflammatory drugs (NSAIDs) is mediated through their inhibitory effects on cyclooxygenase (COX) activity (Mizushima, 2010).

COX is responsible for synthesis of prostaglandin signaling molecules, which are involved in a wide range of physiological processes beyond inflammation. There are two major classes of COX enzymes have which are COX-1 and COX-2. COX1 is constitutively expressed in many tissues and seems to be relevant for the tissue homeostatic functions of prostaglandins, and COX2, which is an inducible form that has a role in many inflammatory and proliferative reactions (Ulrich *et al*., 2006). NSAIDs possess anti-inflammatory and analgesic effects by acting as inhibitors of COX-2 thus reducing the production of prostaglandin that is responsible for hyperalgesic effects (Burian and Geisslinger, 2005).

Despite of their therapeutic effects, NSAIDs are responsible for 21–25% of reported adverse drug events which include immunological and non-immunological hypersensitivity reactions (Kowalski *et al*., 2011). Various side effects have been associated with NSAIDs prescription including gastrointestinal bleeding, ulcers, ulcer complications and ulcer complications leading to death (Wright, 2002). In addition, NSAIDs increased the risk of adverse cardiovascular events for example

congestive heart failure, increase blood pressure, myocardial infarction and ischemia (Risser *et al*., 2009).

2.6 Carrageenan-induced Paw Oedema Model

Animal models of inflammation and pain have been widely used to study the mechanisms of inflammatory pain. There are numerous inflammatory agents or irritants can be used to develop inflammatory animal models including complete Freund's adjuvant, carrageenan, zymosan, mustard oil, formalin, capsaicin, bee venom, acidic saline, lipopolysaccharide, inflammatory cytokines, and sodium urate crystals (Table 2.1) which can results in tissue injury and hyperalgesia in such structures as cutaneous/subcutaneous tissues, joints, and muscles (Zhang and Ren, 2011). As shown in Table 2.2, there are various inflammation and pain model used by researchers to study inflammatory diseases that can provide powerful insights into the possible underlying pathologies of human diseases and finding of potential human therapeutics (Webb, 2014).

Table 2.1 Comparison of cutaneous/subcutaneous inflammatory pain models (Umar Zaman, 2019)

Chemical	Time of Onset	Duration
CFA	2-6 h	1-2 weeks
Carrageenan	1 h	24 h
Mustard oil	5 min	<1 h
Zymosan	30 min	24 h
Formalin phase I	<1 min	5-10 min
Formalin phase II	10 min	1 h
Bee venom	1 min	96 h
Capsaicin	1 min	<1 h

Table 2.1 Typical animal models of inflammation and autoimmune diseases (Webb, 2014)

Name	Disease	Type	Species	Rationale	Strength/advantages	Weakness/disadvantages
Adjuvant induced arthritis	Inflammation/ Rheumatoid arthritis/pain	Joint destruction	Rat (mouse)	Designed for NSAIDs	Highly reproducible NSAIDs work	Limited predictability for other drug classes
Collagen induced arthritis	Rheumatoid arthritis/pain	Joint destruction	Mouse (rat)	Most reflective of human joint pathology	Respond to NSAIDs/TNF inhibitors/IL-1 inhibitors	Not reflective of all human joint pathology-acute disease model, self-limiting, limited predictability for cell signaling based drugs
Endotoxin induced arthritis	Rheumatoid arthritis	Joint destruction	Mouse, rat	Inflamed joints	Respond to NSAIDs	Some aspects of joints disease
Antibody induced arthritis	Rheumatoid arthritis	Acute Joint destruction	Mouse	Reflect acute phase of disease	Respond to NSAIDs. P38 and PDE inhibitors	Model is acute
Carrageenan	Inflammation	Inflammed paw	Mouse	Generalized	IL-1RA, anti-IL-6	Non-specific

paw model	oedema			inflammation	NSAIDs model	
Tail flick	Pain	No treatment required	Mouse, rat	Acute pain (burn)	Very specific for analgesic	Does not reflect neuropathic or inflammatory pain
EAE (EAE)	Multiple Sclerosis	Neural sheath derived antigens	Mouse (guinea pig, rabbit, primates)	Demonstrates relapsing/remitting MS like disease	Can be used with variety therapeutics but not always predictively	Generally a self-limiting disease unlike MS
Endotoxin induced sepsis	Endotoxic shock, systemic sepsis	Primarily acute in susceptible animal species	Mouse, rat, guinea pig, rabbit	Reflects the results of acute bacteremia	Not predictive utility	Some mouse strains are resistant human disease is demonstrably different

Table 2.2. Continued

Name	Disease	Type	Species	Rationale	Strength/advantages	Weakness/disadvantages
Inhaled antigen induced tracheal constriction models	Allergic disease asthma	Tracheal inflammation	Mouse, rat, rabbit, dog, monkey	Shows smooth muscle constriction in trachea and will respond to many anti-asthmatic drugs	Not predictive	No animal exactly mimic human bronchial constriction
Delayed type hypersensitivity models	Skin inflammation, allergy	Skin inflammation	Mouse, rat, guinea pig,	Shows cellular infiltrate and classic DTH	Useful	Can be used for topical treatments for allergic disease
Inflammatory bowel disease (Crohn's disease)	IBD, colitis, Crohn's disease	Autoimmune inflammation and bowel destruction	Mouse	Shows most of the features of Crohn's diseases and ulcerative	Not always predictive although anti-TNF's and some other drugs work here	Not completely reflective of human disease (gut flora differences between mouse and man)

				colitis		
Transgenic mouse models of autoimmune diseases	Many autoimmune/inflammatory diseases	Can show cellular and tissue features that resembles diseases under study	Mouse	Allows detailed study of effects of gene depletion or amplification/mutation in vivo	Can uncover new targets for therapeutic evaluation	Amplifies specific gain or loss effects of specific genes
Humanized mouse models	Primarily used for hematologic studies of various types	Human immune cells can be studied in vivo reflecting some aspects of human immunity	Mouse	Used to probe specific aspects of human immunity that may not be pursued in normal volunteers	Some aspects of human immunity may be studies in an in vivo setting	Not the same physiology as in man

Carrageenan is a generic name for a family of gel-forming and viscosifying polysaccharides, which are obtained by extraction from certain species of red seaweeds (Necas and Bartosikova, 2013). Carrageenan is widely used in processed food including dairy products, processed meats, infant formula, as well as cosmetics and pharmaceutical products where the serves as a thickener, stabilizer, or emulsifying agent (Borthakur *et al*., 2012).

There are three main types of extracted carrageenan, iota (ι), kappa (κ) and lambda (λ) (Figure 2.5) depending on which seaweed it has been extracted from. Kappa carrageenan is extracted from species of Kappaphycus such as *K. alvarezii* and *K. striatum* whereas iota carrageenan is extracted from *Eucheuma denticulatum,* and Lambda carrageenan is primarily extracted from *Chondrus crispus*. Lambda carrageenan that is extracted from *Chondrus crispus* has been used for decades in research for its potential to induce inflammation (Barth *et al*., 2016). According to Radhakrishnan *et al*., (2003), carrageenan is one of the most commonly used irritant to produce short-lasting (less than 24 hours) acute inflammation and hyperalgesia in animal models.

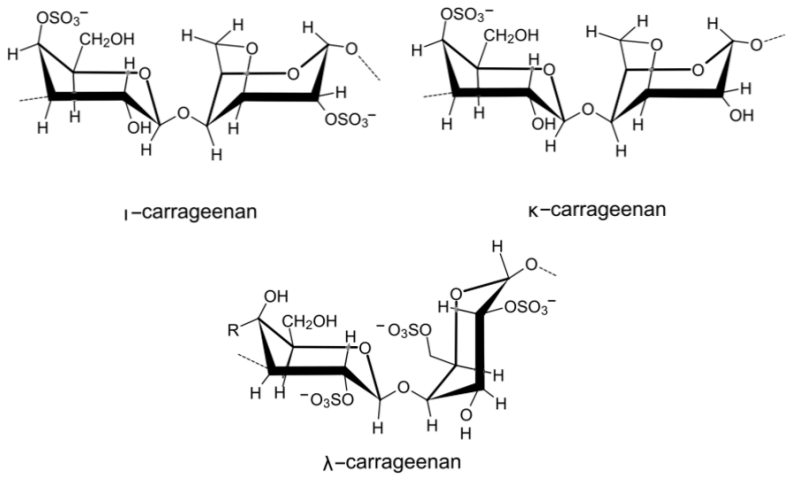

Figure 2.4 Chemical structure of carrageenans (Necas and Bartosikova, 2013)

Studies by Bhattacharyya *et al*., (2008) proves that similar with bacterial lipopolysaccharide, carrageenan also able to interact with TLR4 receptors thus induce the activation of NF-κβ pathways. The carrageenan-induced inflammatory cascades by direct binding to TLR4 thus activate NF-κB pathway (Borthakur *et al.*, 2012). Therefore, intra plantar injection of carrageenan will results in the development of inflammatory response with cardinal signs of inflammation such as redness, heat and local hypersensitivity as well as the releasing of various inflammatory cytokine and chemokine also infiltration of immune cells (Patil *et al*., 2017).

2.7 Free Radical

Free radical are the products of normal cellular metabolism and can be defined as an atom or molecule containing one or more unpaired electrons in valency shell or outer orbit and is capable of independent existence (Bala and Haldar, 2013). The unpaired electron of a free radical makes it unstable, short lived and highly reactive towards chemical reactions with other molecules (Chakraborty and Ahmed, 2011; Kumar and Pandey, 2015). The free radicals are derived from both endogenous sources (mitochondria, peroxisomes, endoplasmic reticulum, phagocytic cells) and exogenous sources (pollution, alcohol, tobacco smoke, heavy metals, transition metals, industrial solvents, pesticides, certain drugs like halothane, paracetamol, and radiation) (Phaniendra *et al*., 2015).

Normally, free radical protects body from bacteria viruses and other foreign substances. When our antioxidant defenses are adequate, damage caused by those free radicals is repaired without many consequences. However when excessive amount of free radicals generates it can damage proteins, lipids, enzymes and DNA that can alter downstream cell signaling and a cause a variety of disease (Khanna *et*

al., 2014). Free radical can be classified into two main groups, reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Sun *et al*., 2017).

2.8 Reactive Oxygen Species

ROS comprise both free radicals and other non-radical reactive species. The examples for the radicals include Superoxide superoxide ($O_2^{\bullet -}$), oxygen radical ($O_2^{\bullet\bullet}$), Hydroxyl (OH•), Alkoxyradical (RO•), Peroxyl radical (ROO•), nitric oxide (nitrogen monoxide) (NO•) and nitrogen dioxide (NO_2•) (Phaniendra *et al*., 2015; Pham-Huy *et al*., 2008). The non-radical species include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hypobromous acid (HOBr), ozone(O_3), singlet oxygen (O_2), nitrous acid (HNO_2), nitrosylcation (NO^+), nitroxyl anion (NO-), dinitrogen trioxide (N_2O_3), dinitrogen tetraoxide (N_2O_4), nitronium (nitryl) cation (NO_2^+), organic peroxides (ROOH), aldehydes (HCOR) and peroxynitrite (ONOOH) (Phaniendra *et al*., 2015).

ROS/RNS role are both beneficial and toxic to the body because at low or moderate levels, ROS/RNS possess beneficial effects on cellular responses and immune function meanwhile overproduction of ROS/RNS will generate oxidative stress, a deleterious process that can damage all cell structure (Pham-Huy *et al*., 2008). Under normal condition, ROS serve several physiological functions, where they involve in signaling pathways that modulate physiological processes such as inflammation, apoptosis, regulation of smooth muscle tone, and leukocyte adhesion to the vascular endothelium (Chakraborty and Ahmed, 2011).

On the other hand, excessive production of ROS/RNS over a prolonged period of time can cause damage to the cellular structure and functions when they oxidize protein, lipid cellular constituents and damage the DNA due to high oxidizing ability (Mittal et al., 2014). In addition, ROS may induce somatic

mutations, preneoplastic and neoplastic transformations (Hussain *et al*., 2016). Therefore, ROS play an important role in diverse range of degenerative diseases for example atherosclerosis, inflammatory joint disease, asthma, diabetes, kidney diseases, and degenerative eye disease as well as various cancers due to damaged DNA (Biswas *et al*., 2017; Yang *et al*., 2018).

The source of ROS including enzymatic reactions in various cell compartments, including the cytoplasm, cell membrane, endoplasmic reticulum (ER), mitochondria, and peroxisome, as part of basal metabolic function. They are also generated specifically by enzymes such as NOXes (nicotinamide adenine dinucleotide phosphate [NADPH] oxidases) and serve a signaling function in the cell (Forrester *et al*., 2018).

Generally, ROS are involved in the initiation, progression and resolution of inflammatory response (Chelombitko, 2018). This is because epithelial cells, resident macrophages, endothelial cells, and recruited inflammatory cells, such as neutrophils, eosinophils, monocytes, and lymphocytes, produce ROS at the site of inflammation by their phagocytic activity (Lee and Yang, 2012). In addition, increasing of NADPH oxidases by pro-inflammatory cytokines, such as tumor necrosis factor-a (TNF-α) and interleukin-1β (IL-1β) during infection will results in increasing of ROS that particularly important as a host defense mechanism but excessive NADPH oxidase activation has also been implicated in oxidative stress (Fischer and Maier, 2015; Yang *et al*., 2007). Furthermore, ROS can activate NF-κβ in response to inflammatory agonists Forrester *et al.,* 2018).

2.9 Antioxidant

Antioxidants are enzyme that reduces the level of ROS/RNS thus counteract the overproduction of ROS/RNS (Lei *et al*., 2015). Antioxidants existed in many

dietary natural sources such as vegetables, fruits, and beverages and dietary antioxidants such as polyphenols and flavonoids thus can help reducing oxidative stress on cellular structure and prevent oxidative damage (Yeung *et al*., 2019; Zhang and Tsao, 2016). Oxidative stress is a cellular phenomenon or condition which occurs as a result of physiological imbalance between the levels of antioxidants and oxidants (free radicals or ROS/RNS) where the level of antioxidants have been overwhelmed by the free radicals due to excessive production of reactive species (Ighodaro and Akinloye, 2018).

It is important to keep balance between oxidants and antioxidants in order to protect cells from oxidative damage that can lead to many chronic diseases, such as cancer, diabetes, cardiovascular disease and many more (Zou *et al*., 2016). Antioxidants inhibit the oxidation reaction of free radicals by exchanging one of their own electrons with the free radical molecules to stabilize them (Sanchez, 2017). Antioxidants can be endogenous and dietary antioxidants such as polyphenol, vitamin A for example carotenoids, vitamin E (α-tocopherol), β-glucan, proteins, ascorbic acid (vitamin C), glutathione and many more (Sanchez, 2016).

When endogenous antioxidants are inadequate to remove free radical from the body, it becomes important for the body to receive exogenous natural antioxidants such as phenolic compounds, secondary plant metabolites that are found naturally in all plant materials, including plant based food products (Grzesik *et al*., 2018). Study has found that phenolic and flavonoid compounds act as antioxidants that exhibits anti-allergic, anti-inflammatory, anti-diabetic, antimicrobial, anti-pathogenic, antiviral, antithrombotic, and vasodilatory effects due to their ability to protect against oxidative diseases, activate or inhibit various enzymes bind specific

receptors, and protect against cardiovascular diseases by reducing the oxidation of low-density lipoproteins (Huyut *et al*., 2017).

2.10 Vitex rotundifolia

V. rotundifolia is an important coastal and medicinal plant, also known as Beach Vitex , a deciduous, sprawling shrub with round leaves and spicy fragrance that is widely distributed in coastal areas of Japan, Southeast Asia, Pacific Islands, and Australia (Nigam *et al*., 2018; Chaudhry *et al*., 2019; Sun *et al*., 2019). *V. rotundifolia* belongs to the Verbenaceae family of angiosperms, also placed in Lamiaceae family, is a low-growing, salt tolerant, shoreline/sea side shrub (Cousins *et al*., 2017; Parkhe and Bharti, 2019; Kim *et al*., 2020). The diameter of *V. rotundifolia* is around 6-8 feet and 2 inches feet where the leaves are 1-2 inches long and round with gray-green to silvery color and has spicy fragrance (Rani and Sharma, 2013). *Vitex rotundifolia* grows 0.5-1.0 m in height; however growth is primarily concentrated in a dense mat horizontally, with spreading branches up to 20 m long and approximately 5 m in width and nodal rooting of the branches contributes to this mat-like growth (Banisteria, 2009).

Various phytochemical constituent (Figure 2.6) can be found in this plants including flavonoids, alkaloids, saponins, iridoids, phenolics, mono- and diterpenes, α-pinene, α-terpineol, 1,8-cineole and manoyl oxide, phenylnapthalene; polymethoxyflavonoids, dehydroabietane, biformene, rotundiferan, vitexicarpin, prerotundifuranne and rotundifuranne, aucubin, thunbergol, mussaenosidic acid, trans-phytol and sabinene that act as cytotoxic, cell-cycle arrest, apoptosis inducer, antioxidant, activation c-Jun N-terminal kinase (JNK), inactivation of NF-κB, and caspase3 activation (Kim *et al*., 2014; Nigam *et al*., 2018).

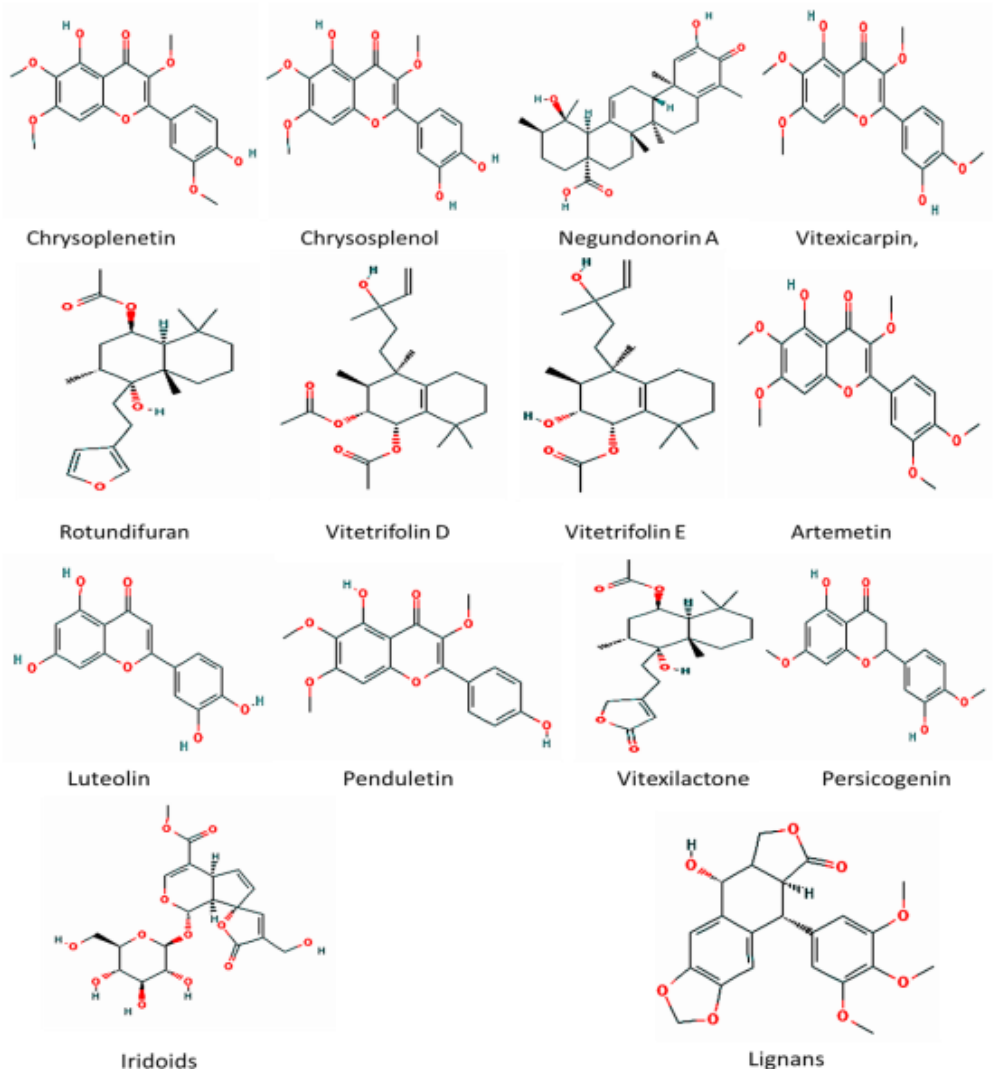

Figure 2.5 Phytochemical constituents of *Vitex* spp. (Nigam *et al*., 2018)

2.10.1 Medicinal use of *V. rotundifolia*

The presence of phytochemical constituents and bioactive compounds that exhibits various therapeutic activities determines the medicinal properties of plants (Mohammed *et al*., 2016). *V. rotundifolia* is has been used as folks medicine because most parts of Vitex plants (the leaves, fruits, roots and stems) (Figure 2.7) are known to have many medicinal values (Abdul Hakeem *et al*., 2016). *Vitex* spp. has been used as traditional medicine to treat diarrhoea, gastrointestinal disorders, sprain,

rheumatic pain, inflammation, cancer, respiratory infections, migraine premenstrual problems, depression, allergy, wounds (Chan *et al*., 2018); Abdul Hakeem *et al*., 2016;). Moreover, *V. rotundifolia* possessed potent repelling activity, stronger antioxidative activity, antiproliferative activity, potential chemopreventive agents, antiaging and skin-whitening, antipyretic, analgesic, anti-inflammatory functions, bacteriostasis and antimalarial activity (Rani and Sharma, 2013).

2.10.1 (a) Leaves

The leaves of *V. rotundifolia* produces thick, waxy cuticle 1-2 inches long and round with gray-green to silvery color and has spicy fragrance containing large amounts of diverse n-alkanes where these compounds are transferred to the surface of sand particles where they cause intense hydrophobicity in the substrate (Cousins *et al*., 2017; Rani and Sharma, 2013). Leaves of *V. rotundifolia* have been used as insects repellent due to the present of rotundial that act as insect repellent that are more powerful than deet (Cousins *et al*., 2017). Methyl-p-hydroxybenzoate from the leaf of *V. rotundifolia* exhibited 100% mortality against the larvae of of *Culex quinquefasciatus* and *Aedes aegypti* explained the effectiveness of the leaves as mosquitoes repellent (Chan *et al*., 2016).

Figure 2.6 Leaves, flower, and fruits of *V. rotundifolia* (Chan *et al*., 2016).

2.10.1 (b) Roots

V. rotundifolia roots characteristics is nodal rooting that allows the plant to form dense mats that spread from mother plants to distances of more than 10 m and roots and stems are capable of rapid regeneration (Cousins *et al*, 2010). The roots of *V. rotundifolia* are used to treat febrifuge, cough and fever while the stems of *V. rotundifolia* was proven to be very toxic against cultures of several cell line and the aerial parts of this plant are useful in diabetes treatment (Abdul Hakeem *et al*., 2016).

2.10.1 (c) Stems

Young stems of *V. rotundifolia* are square and green or purple, fleshy at the tips, and as the stems mature, the stems develop into round, brown, and woody where the bark will crack and fissure with age while the branches from running stems are upright (Cousins *et al*., 2017). The stems of *V. rotundifolia* has been found to exhibits cytotoxic effects against cultures of several cancer cell lines as well as an important anti-feeding activity against the insect pest *Spodoptera frugiperda* (Meena *et al*., 2011). Leaves and bark of *V. rotundifolia* contain flavones, artemetin, essential oil, 7-dimethyl artemetin, friedelin and some alkaloids and non-flavonoids (Parkhe *et al*., 2019).

2.10.2 Fructus viticis of *V. rotundifolia*

The fruit of *V. rotundifolia* (also known as Fructus viticis) are small succulent drupes, globular or ovoid of 0.2-2 cm size (usually smaller than 2 cm), with hardened endocarp that is divided in four pyrenes, each one with a seed and the ripe pulp of these fruits is characterized by dark-purple to brown colors (Pío-León *et al.,* 2011). It has been used as traditional medicinal plants for the treatment of anemopyretic cold,

headache, rheumatism, cancer and premenstrual disorder (Fang et al., 2016). Moreover, Fructus viticis has been used as traditional Chinese medicine with analgesic properties to provide efficient relief of migraine (Wen *et al*., 2019).

Studies found that Fructus viticis substantiated the anti-tumor properties in diverse range of human cancer cells and their xenograft models in mice due to presence of large amounts of flavonoids, including casticin, luteolin, apigenin, isoorientin, hesperidin, isovitexin and so on (Cao *et al*., 2016). Other medicinal uses of Fructus viticis including alleviating cold-heat between tendons and bones, damp arthralgia and muscular spasms, eyesight-improving, teeth-strengthening, nine-orifices benefiting, expelling white worms, losing weight and delaying senility, eliminate the symptoms of eye-swelling, eye-itching and eye-drying, delay fatigue, alleviating pyrexia, eyes and gums pain, giddiness and headache, used as used for sedation, relieving pains and diminishing inflammation (He *et al*., 2019).

2.10.3 Pharmacology use and bioactivity of Fructus Viticis

Several kinds of compounds, including diterpenoids, flavonoids, lignans, iridoid and phenolic glucoside can be found in the fruits of *V. rotundifolia* (Fructus viticis) (Wang *et al*., 2014). Bioactive compound of Fructus viticis exhibits various pharmacology activities such as anti-cancer, anti-inflammatory, anti-allergy, antioxidants, anti-nociceptive, and antibacterial (Cousins *et al*., 2017). Fructus viticis contains flavonoids such as artemetin, quercetagetin, 5,3′-dihydroxy-6,7,4′-trimethoxyflavanone and casticin, where casticin is the primary active compound, and many researches has been conducted to study chemical constituents of the extract of this fruits and the molecular mechanisms responsible for its effects (Bae *et al*., 2012).

Casticin (3',5-dihydroxy-3,40 ,6,7-tetramethoxyflavone) also known as vitexicarpin or casticine, has shown anti-cancer properties, anti-inflammatory properties as well as anti-asthmatic, tracheospasmolytic, analgesic, anti-hyperprolactinemia, immunomodulatory, opioidergic, oestrogeni,, anti-angiogenic, antiglioma, lung injury protection, rheumatoid arthritis amelioration and liver fibrosis attenuation activities (Chan *et al*., 2018).

Polymethoxyflavonoids of Fructus viticis (vitexicarpin, luteolin and artemetin) were found to induce apoptosis in human myeloid leukemia cells (Chaudhry *et al*., 2019). In addition, diterpenoids that were isolated from Fructus viticis has significantly inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells thus involves in anti-inflammatory activities (Yao *et al*., 2016). Furthermore, previous studies has found that casticin could suppress the inflammatory effect by blocking the NF-κB and MAPK pathways in LPS-induced RAW264.7 macrophage cells and decreases the levels of eotaxin and reduces eosinophil migration in IL-1β-stimulated A549 human lung epithelial cells (Liou and Huang, 2017).

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Chemicals

Chemicals used in this study are shown in **Table 3.1** below.

Table 3.1 Chemicals used in this study

Chemicals	Brands
Fructus Viticis methanolic crude extract	Extracted and processed by Universiti Malaysia Terengganu (UMT)
λ-carrageenan	Sigma-Aldrich, UK
Sodium Pentobarbital	20% Dorminal, Holland
10 % Buffered Formalin	Leica Biosystem Richmon
Ethanol	HmbG Chemicals
PBS	Oxoid, UK
Saline	-
Butylated hydroxytoluene (BHT)	Sigma-Aldrich, UK

3.1.2 Apparatus

Apparatus used in this study were 25 G (1 inch) and 27 G (1/2 inch) needle, 1 cc/mL and 3 cc/mL syringe, analytical weighing scale, dissecting set, gloves, masks.

3.1.3 Instrument

Rat-tail cuff blood pressure measuring system (IITC Life Science, Mouse Rat Blood Pressure (MRBP) System), EDTA vacutainer tube, Randall-Selitto analgesio tester (Ugo Basile Analgesy-Meter).

3.2 Animals

Animal ethics was approved by the USM Institutional Animal Care and Use Committee (USM IACUC) with the ethical approval number:

USM/IACUC/2019/(117)/(983) (Appendix A). There were 30 male Sprague-Dawley (SD) rats supplied by the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia, Health Campus, Kelantan. Animals were received at 6 weeks old, their weight ranged between 140-190 g. The rats have been acclimatized in holding facility for 14 days before starting the experiment. The rats were placed in pair inside a cage in a well-ventilated room. The rats were maintained under standard laboratory condition of temperature between 24-28°C, relative humidity 60-70% and 12-hour light/dark cycle and were given standard commercial pellet diet and water *ad libitum*. The experiment was conducted when the rats at 8 weeks old, weighing between 180-250 grams.

3.3 Experimental design

Based on previous study by Gainok *et al*., (2011); Santos Nogueira *et al*., (2012) and Rivat *et al*., (2002), carrageenan-induced inflammatory pain and nociception test using Randall-Selitto test has been conducted within time interval until 24 hour of testing. Recommendation of total animal number in each test group should be 6-8 animals per group in order to produce valid and precise data and also to reduce possible variability of pain measurement in each animal (Santos Nogueira *et al*., 2012).

Table 3.2 Design of treatment and group of animals

Group	Treatment	No of Animals (n)
A	Vehicle-DMSO (50 µl) + Saline (100 µl)	6
B	Vehicle-DMSO (50 µl) + 2% λ-Carrageenan (100 µl)	6
C	50 mg/mL Extract (50 µl) + Saline (100 µl)	6
D	50 mg/mL Extract (50 µl) + 2% λ-Carrageenan (100 µl)	6
E	100 mM LNMMA (50 µL) + 2% λ-Carrageenan (100 µl)	6
		Total=30 animals

3.4 Carrageenan-induced inflammation and treatments

3.4.1 2% λ-carrageenan solution

Carrageenan solution was prepared by mixing 0.2 g of λ-carrageenan in 10 mL of distilled water to produce 10 mL of 2% λ-carrageenan. The mixture was fully dissolved in 65°C of water bath until the solution become soluble and no clump. The solution was kept in room temperature before using and can be kept up to 6 months.

3.4.2 Plant extracts

Treatment of 50 µL of extract is needed for each rats thus to ensure the treatment is sufficient, 3000 µL of treatment was prepared. In order to prepare 3000 µL of 50 mg/mL of treatments, 150 mg of extract was diluted in 3000 µL of Dimethyl Sulfoxide (DMSO) and was mixed well until fully dissolved.

3.5 Animals experimentation

In vivo experiment was started by measuring all the basal reading of the rats including blood pressure, paw thickness as well as pain threshold prior to treatment and inflammation creation using carrageenan injection. For anesthetic agents, 20g/kg of sodium pentobarbital was given (i.p.) and once animals were fully anesthetized, 50 µL of treatment either Vehicle (DMSO), 50 µL of 50 mg/mL extract or LNMMA (NG-monomethyl-L-arginine) were administered by injection, subcutaneously (s.c) into intra plantar of right hind paw (ipsilateral). The treatment should be given 30 minutes prior to the induction of inflammation thus 100 µL of 2% λ-Carrageenan was injected subcutaneously (s.c.) into ipsilateral 30 minutes after administration of treatment to initiate inflammatory response.

3.6 Blood pressure

Blood pressure was taken to ensure the pain caused by carrageenan-induced paw oedema only results in peripheral and localized pain and not central and systemic pain (Necas and Bartosikova, 2013). Blood pressure of the animals was measured using Mouse Rat Blood Pressure (MRBP) System with non-invasive tail cuff method (Figure 3.1). Systolic reading (Figure 3.2) was taken triplicates at predetermined time (basal, 30 minutes, 2,6,12 and 24 hours post-carrageenan/saline injection) to reduce stress experienced by the animals. During the habituation, the animals were acclimatised with the restrainer for a week to familiarise the animals with the procedure. The restrainer was covered with a cloth to make them calmer during the reading process.

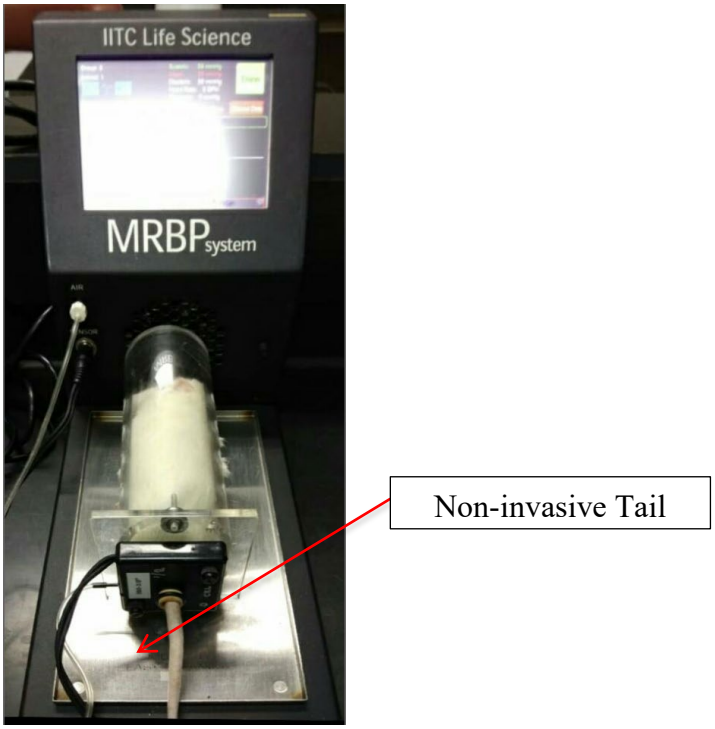

Figure 3.1 Blood pressure measurement by using tail-cuff method MRBP system

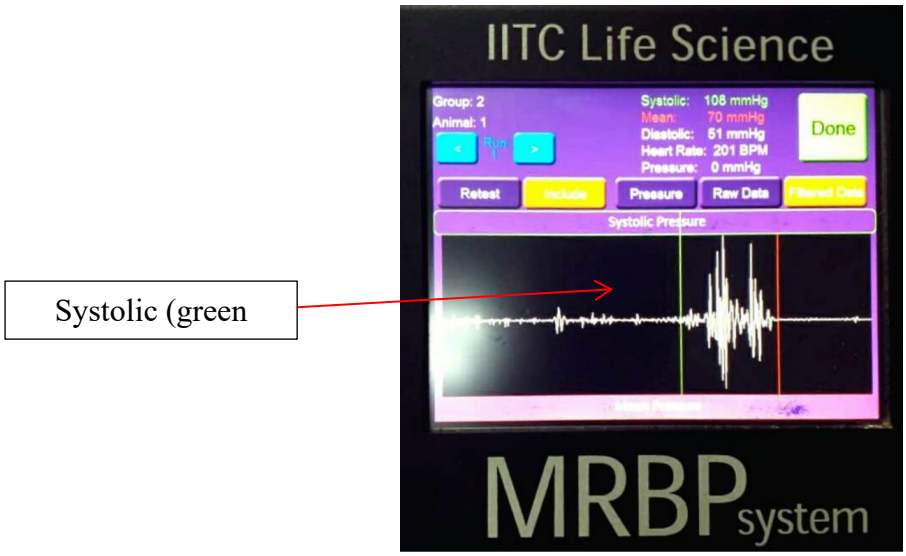

Figure 3.2 Systolic reading of MRBP system

3.7 Paw oedema measurement

Paw swelling or oedema is one of the cardinal sign of inflammation thus measuring the paw thickness. It is a useful method in assessing inflammatory response as well as examining the ability of the treatment to reduce or prevent the development the carrageenan-induced paw oedema (Sarkhel, 2016). Paw oedema of the rats were measured using digital vernier caliper with accuracy of ± 0.2 mm/0.01 at predetermined time (Basal, 30 minutes, 1, 2, 4, 6, 8, 12, 24 hours) post-carrageenan/saline injection according to assigned group. The rats were restrained properly by gripping the back skin firmly and securely to reduce stress thus animals can remain calm throughout the procedure. The measurement was taken by placing the vernier caliper around the paw oedema between the bump as shown in Figure 3.3 and the procedure was repeated 3 times for each rat to produce triplicate reading.

Figure 3.3 Measurement of paw oedema using digital vernier caliper

3.8 Randall-Selitto Mechanical Hyperalgesia test

Due to the releasing of various inflammatory mediators during inflammation, the sensitivity to pain increased and caused hyperalgesia. Various methods can be used to assess central antinociceptive and peripheral activity in animals and of the method is Randall-Selitto test (Porto et al., 2013) and in this experiment, we used the Ugo Basile Analgesy-Meter (Figure 3.4). Randall-Selitto is an instrument that has been described as the most predictive of animal models of acute pain (Gainok *et al*., 2011). Randall-Selitto or paw pressure test was developed as a tool to assess response thresholds to mechanical pressure stimulation and is often considered a measure of mechanical hyperalgesia (Deuis et al., 2017). For this experiment, pressure was applied to the ipsilateral until the animal withdrew or vocalized pain, at which the weight applied during paw withdrawal was recorded. The maximal amount of pressure withstood by the animal was limit to 200 mg to avoid any injuries or tissue damage to the paw which could cause severe pain to the animal. The measurement was taken at predetermined time (basal reading, 30 minutes and at 1, 2, 4, 6, 8, 12, 24 hours post carrageenan/saline injection) for 3-4 times at one specific time with the animal was rested for a minute between each reading

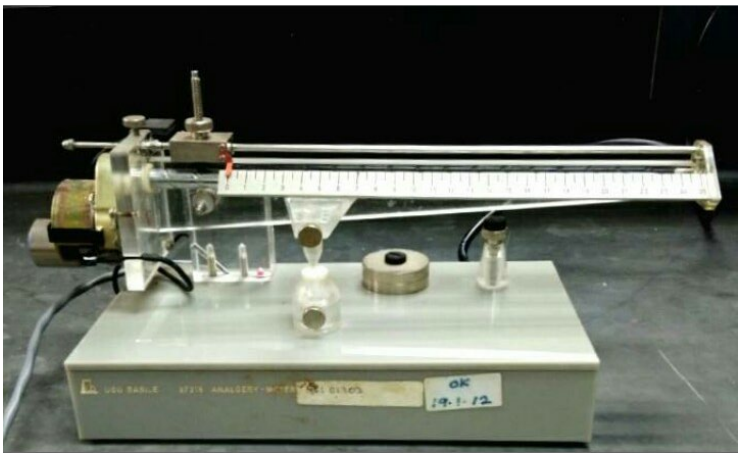

Figure 3.4 Ugo Basile Analgesy-Meter Randall-Selitto test

3.9 Anaesthesia and Euthanasia

Sodium pentobarbital has been used as anaesthetic agents in this study. For anaesthesia, 200 mg/kg of sodium pentobarbital stock solution was diluted using DMSO to produce 20 mg/kg of working solution (calculation shown in Appendix B). Intra-peritoneal (i.p) injection of 20 mg/kg of sodium pentobarbital prior to treatment and carrageenan injection into the rat hind paw able to produce 20-35 min of anesthesia. The rats were monitored accordingly after the injection of anesthesia to ensure there is no excessive of cardiac and respiratory functions or insufficient anesthesia. After 24 hours post carrageenan injection the animals were euthanized using 60 mg/kg sodium pentobarbital that was prepared from stock solution 200 mg/kg. The animals were injected 60 mg/kg of sodium pentobarbital intra-peritoneally (i.p) and the animals were monitored to confirm death. The death of the rat was confirmed by assessed by pedal reflex in which the paw of the rat was pinched firmly to ensure the rat was no longer experience pain. The heart beat and the respiratory pattern also were monitored to confirm death before the cardiac puncture and tissue collection can be made.

3.10 Haematology (Full blood count)

To further investigate the effects of carrageenan and treatment on immune cell, blood sample of each rats after 24 hours was collected and sent to BP Laboratory Sdn Bhd. Approximately 5 mL of blood was withdrawn from cardiac puncture and was transferred immediately into EDTA vacutainer blood collection tubes. Once transferred to the tubes, the tube was tilted to ensure the blood will not clot. Three blood samples (n=3) from each group were collected and were sent to B.P Clinical Lab Sdn Bhd Kota Bharu, Kelantan, Malaysia for full blood count (FBC) analyses.

3.11 Sample collection

Once the death of the rat was confirmed, the experiment was preceded with ipsilateral paw sample collection. The paw sample was harvested by slicing the paw's skin with scalpels. Figure 3.5 shows the dissecting area of the rat hind paw. Three harvested samples (n=3) from each treatment group were kept in cryotubes and stored at -80^{o}C for nitric oxide (NO) and TNF-alpha determination in paw while the other three samples (n=3) from each group were fixed into 10% buffered formalin for histology analysis

Figure 3.5 Dashed line is the area of paw tissues dissection

3.12 Antioxidant Assay Using DPPH Free Radical Scavenging

DPPH free radical scavenging assays allow the assessment of free radical scavenging ability of plant extracts (Rahman *et al*., 2015). The ability of the plant extract to donate hydrogen atom was determined by the decolorization of methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH produces violet/ purple color in methanol solution and fades to shades of yellow color in the presence of antioxidants. DPPH solution was prepared by diluting 0.003 g of DPPH in 100 mL of methanol and 2.4 mL of this solution was mixed with 1.6 mL of Fructus Viticis methanolic crude extract in 96 well plates. Samples were incubated in the dark for approximately 30 minutes. The changes of colour from purple to yellow was determined by using spectrophotometer at wavelength 517 nm as shown in Appendix C. Absorbance decline by DPPH solution was used as an indication for high antioxidant activity. The percentage antioxidant activity was calculated using the given formula:

%DPPH scavenging = $[(A_0 - A_1)/ A_0)]$ x 100

A_0= absorbance of the control, A_1= absorbance of the extract together with DPPH. Butylated hydroxytoluene (BHT), Gallic acid, Quercetin and Ascorbic acid were used as standard and $IC50$ value of each extract was measured. Comparison of antioxidant of methanolic crude extracts of Fructus Viticis was made.

3.13 Statistical Analysis

All data are presented as mean value ± standard error mean (S.E.M). Comparisons of all data between groups were done using Graph Pad Prisms Version 7. Paw oedema, pain and blood pressure data were analyzed using two-way ANOVA. Meanwhile, full blood count analysis and antioxidant activity was

analyzed by using one-way ANOVA with multiple comparison where is appropriate. P values of <0.05 was considered significant different between group.

3.14 Chemicals, apparatus, instrument and procedures that will be conducted/ Not Complete

3.14.1 Chemicals

Table 3.3 Chemicals that will be used in this study

Chemicals	Brands
Xylene	Merck
Ethanol	HmbG Chemicals
Paraffin wax	Leica Biosystem Richmond
Hematoxylin	Merck
Eosin	Merck
DPX	R & M Marketing Essex, UK

3.14.2 Apparatus

Gloves, masks, slides rack, coplin jar, dropper and glass slide

3.14.3 Instrument

Instrument that will be used are tissue processor (Leica, U.S), paraffin dispenser, cold plate, hot plate, rotary microtome, light microscope and water bath.

3.14.4 Histopathology

Paw tissues that were harvested from three animals for each group (n=3) was fixed in 10% buffered formalin for at least 48 hours and stored at room temperature. Tissues will be dehydrated in a series of alcohols before being cleared in xylene and infiltrated with paraffin at 60^{o}C by using paraffin dispenser. The tissue will be processed by using tissue processor for overnight by using automated tissue processor (Leica, U.S). Blocks will be sectioned with thickness of 4 μm using rotary microtome and sections will be mounted onto Mayer's albumin coated glass microscope slides. The sections will be stained with hematoxylin and eosin (H&E) where hematoxylin will stain cell nuclei and eosin will stain most of the cytoplasmic components thus allow the differentiation of various immune cells. Staining procedure starting by submerging the sides into haematoxylin solution for 6 minutes then wash with water for 5 minutes. Then immerse the slides into 0.1% acid alcohol solution to decolorize and wash again in running tap water for 5 minutes. Next, the slides will be immersed in 1% eosin for 2 minutes. Dehydrate the slides by immersing it into absolute ethanol (100 % ethanol). The slides will be submerged into absolute xylene for 4 minutes before observing under a light microscope and finally a coverslip is placed on slide and mounted using DPX mounting medium.

3.14.5 Quantify infiltrated cells using Image-J software

Quantification of infiltrated cells to the paw oedema will be done using Image-J (National Institutes of Health and Laboratory for Optical and Computational Instrumentation (LOCI), University of Wisconsin)**.** This software allows user to turn images into quantifiable data. All original photomicrographs (RBG format) of hematoxylin and eosin stained paw tissues sections at 10X magnification will be

analyzed. The quantity of infiltrated inflammatory cells will be quantified at the specific area.

3.14.6 Determination of Nitric Oxide in Paw Tissues using Griess Assays

The harvested ipsilateral paws from three animals for each group (n=3) will be used for assessing nitric oxide production using Griess method. On the day of processing, the harvested paws that were kept in cryopreserved vial tube and stored in -80^{o} will be weighed individually for 100 mg before placing in 500 µL of sterile phosphate buffer solution (PBS), pH 7.4 (Gautam *et al*., 2014),. Paws tissue will be homogenized using electrical homogenizer followed by centrifugation at 3000 rpm for 15 minutes and the supernatant will be used for final processing. 100 µL of supernatant will be taken in each well of a 96 well plate will followed by 100 µL of Griess reagent. The plate then will be incubated in a dark for 15 minutes and the presence of NO in each sample will be read at wavelength of 540 nm using UV-Spectrophotometer The reading of each sample then will be used to determine the concentration of NO based on NO standard curve and for converting absorbance readings to nitrite concentration.

3.14.7 Statistical Analysis

All data will be presented as mean value ± standard error mean (S.E.M). Comparisons of all data between groups will be done using Graph Pad Prisms Version 7.Comparison of nitric oxide measurement and quantification of inflammatory cells in tissue will be analyzed by using one-way ANOVA with multiple comparison where is appropriate. P values of <0.05 will be considered significant different between group.

CHAPTER 4

RESULTS

4.1 Gross Observation of Paw Oedema

Initially all rats showed normal paw appearance with no sign of inflammation such as redness, heat, oedema and pain with average of basal reading of paw thickness 4.9 ± 0.06 mm. At the beginning of vehicle+saline injection, all rats in the group developed minor oedema due to the introduction of needles into the hind paws and resolved to normal size after 24 hours as shown in Figure 4.1 (B). As expected animals injected with DMSO+2% (w/v) λ-carrageenan developed massive oedema with clear cardinal sign of inflammation which are redness, heat, swelling and sign of pain even after 24 hour post injection as shown in Figure 4.1 (C). Despite of that, rats that were treated with Fructus viticis extract (50 mg/mL extract + carrageenan) seems to have delay in the oedema development compared DMSO + carrageenan treated group which developed severe oedema. In addition, animals that received 50 mg/mL extract + carrageenan treatment shows less severe inflammatory sign compared to carrageenan group as shown in Figure 4.1 (E). Meanwhile, LNMMA + carrageenan treated rats also developed massive oedema which is comparable to DMSO + carrageenan group (Figure 4.1 (F).

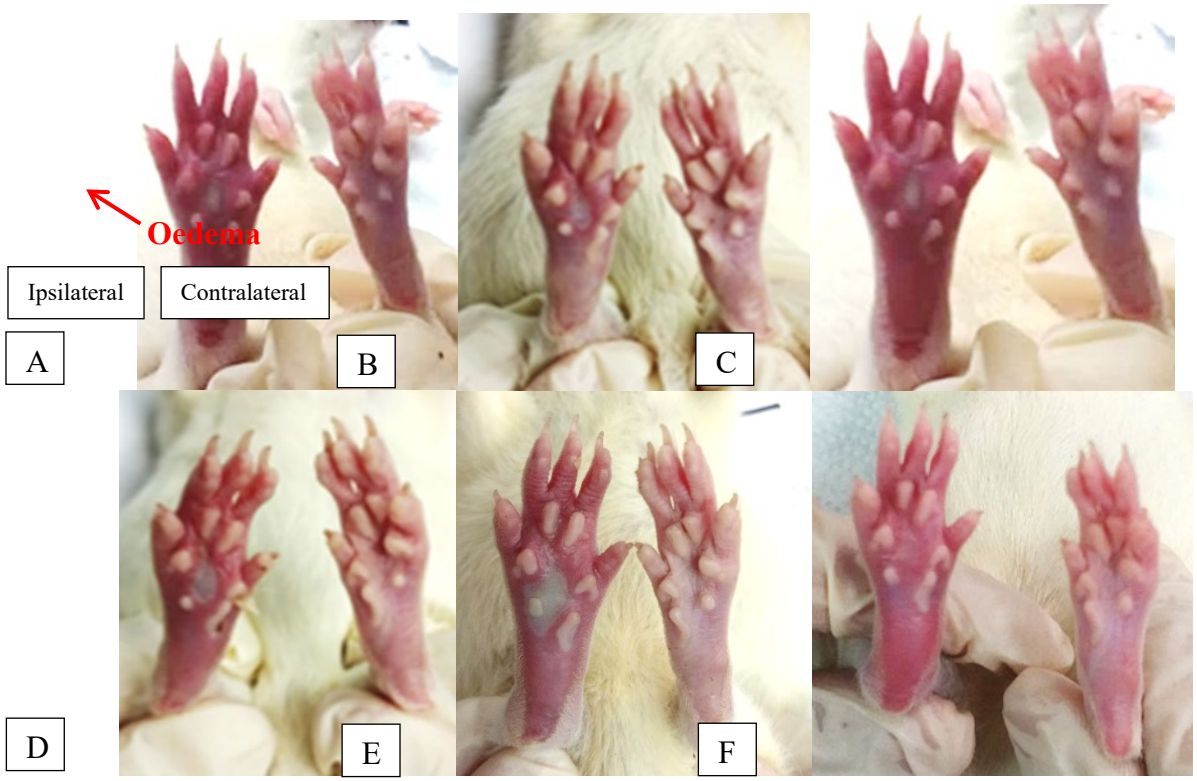

Figure 4.1 Gross observation of rat hind paws at 24 hours post injection with treatments. (A) Comparison between ipsilateral (injected) and contralateral (non-injected) paws. (B) Ipsilateral paw of vehicle + saline. (C) Ipsilateral paw of DMSO + 2% λ- carrageenan showing intense inflammation manifested by severe oedema. (D) Ipsilateral paw of 50 mg/mL extract + saline. (E) Ipsilateral paw of 50 mg/mL extract + carrageenan showing less inflammation. (F) ipsilateral paw of LNMMA + carrageenan.

4.2 Paw oedema

In this study, the development of paw oedema after treatment/carrageenan injection was investigated for 24 hours. Results revealed that the measurement of normal paw thickness of all rats before injection of treatment/carrageenan was in average of 4.2-5.4 ± 0.06 mm Generally, most of paws were having swollen paw after 30 minutes of vehicle/treatment+saline/carrageenan injection which due to small injury caused by injection needle and administration of fluid (150 ul). Paws treated with DMSO+saline started to resolved from 1 hour (6.3 ± 0.4 mm) and the paw thickness was maintained until 24 hours (5.5 ± 0.3 mm) with no obvious cardinal signs of inflammation as shown in Figure 4.2. In this study, Fructus viticis crude extract that was used as treatment did not cause any significant inflammation with no significant alteration of paw oedema at all-time point when compared to control (DMSO+vehicle) as shown in Figure 4.2. As expected, injection of 2% λ-carrageenan has produced a massive oedema which manifested by the increasing of paw thickness at all-time points until 24 hours. The paw thickness injected with carrageenan increased almost 50% at 24 hours (8.4 ± 0.2 mm; $p<0.0001$) when compared to the basal reading (5.0 ± 0.1 mm). Interestingly, in this study, we have demonstrated that rats that received 50mg/ml of Fructus viticis crude extract prior to carrageenan injection has showed a significant ($p<0.01$) delayed of oedema development in paws when compared to paw of rat injected with DMSO+Carrageenan at 4 hours (Extract+Carr: 8.7 ± 0.2mm vs DMSO+Carr: 9.9 ± 0.2 mm) and 6 hours (Extract+Carr: 8.7 ± 0.3 mm vs DMSO+carr: 9.9 ± 0.2 mm). However, after 8 hours to 24 hours there is no significant effect of anti-inflammatory activity of the extract when compared to the DMSO+carrageenan group as shown in Figure 4.2 and Figure 4.3.

LNMMA is nitric oxide synthase inhibitor that is expected to reduce the development of inflammatory mediator nitric oxide (NO). However, in this study, we have demonstrated that LNMMA did not cause any significant reduction of paw oedema caused by the carrageenan. There was no significant difference when compared to DMSO + carrageenan group as shown in Figure 4.2 and Figure 4.3.

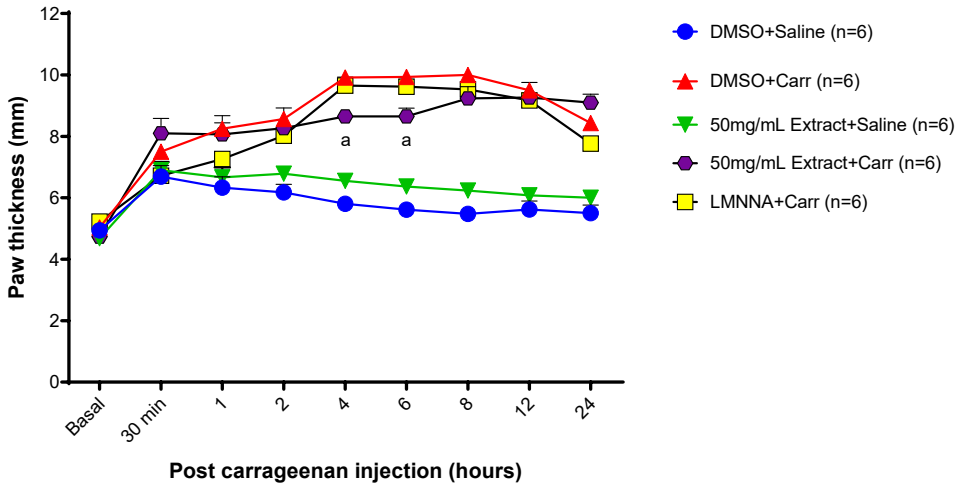

Figure 4.2 Comparison of mean paw thickness (mm) of the rats treated with different treatment at different time interval for 24 hours. Paw oedema was measured using digital vernier caliper. Carrageenan injection has results in enormous paw oedema at all-time point when compared to saline group. Extracts exhibits anti-inflammatory effect by delaying the paw oedema and significantly ($^{a}P<0.01$) different at 4 hours and 6 hours when compared to DMSO + carrageenan group (n=6).

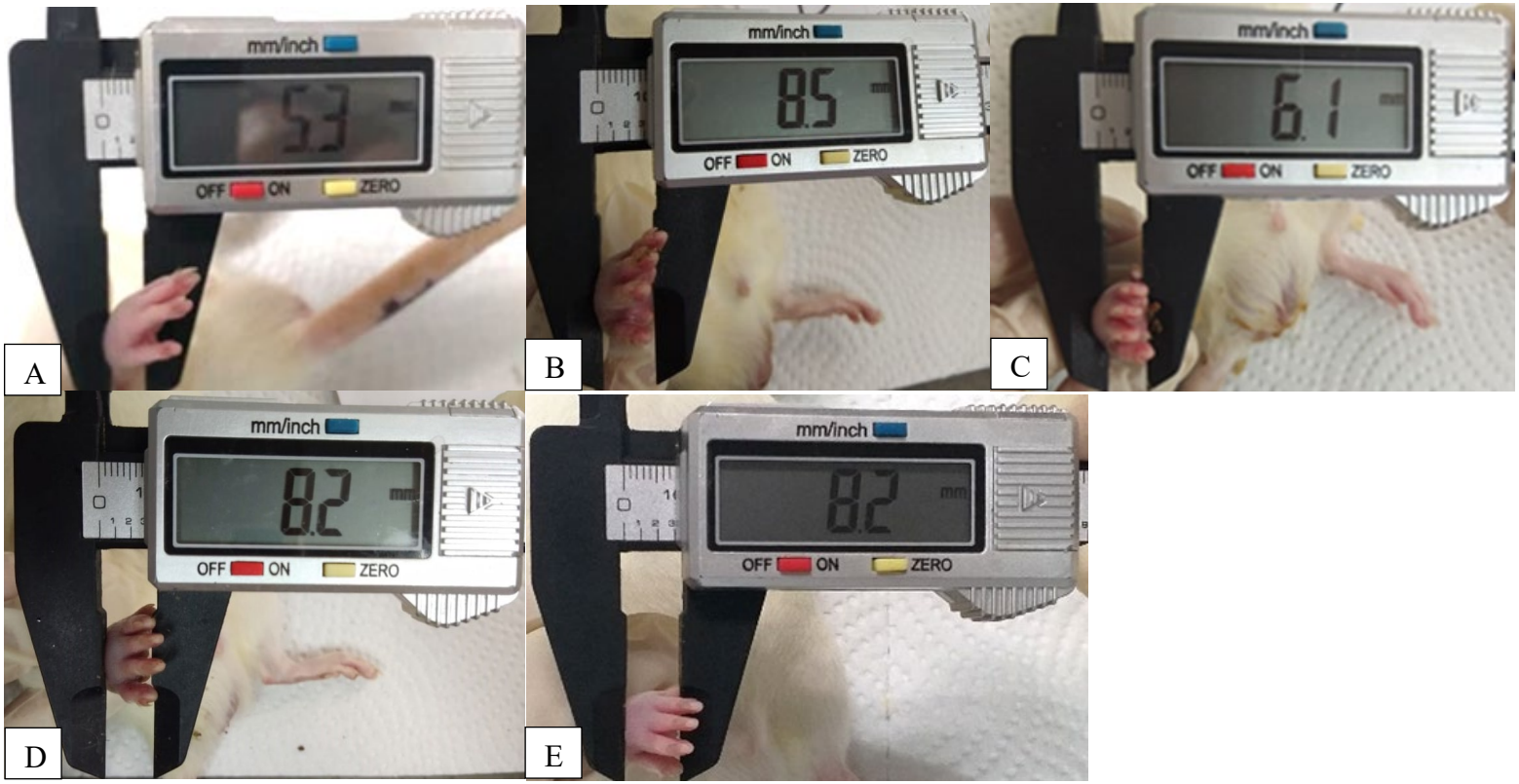

Figure 4. 3 Representative of paw thickness measurement at 24 hours post treatments injection by using digital vernier caliper; A: DMSO + saline, B: DMSO + carrageenan, C: 50 mg/mL extract + saline, D: 50 mg/mL extract + carrageenan, E: LNMMA + carrageenan.

4.3 Pain behaviour

Apart of evaluating the oedema development, in this study, the effect of carrageenan/treatment to the development of pain (nociception) in the hind paw was also investigated. Generally, the measurement of mechanical nociception threshold for all rats before experimentation was ranged from 107.5-200.0 g (168.8 ± 5.4 g).

At the beginning of vehicle/treatment+saline/carrageenan injection, the rats probably experienced pain from the introduction of the needle thus all rats showed reducing in mechanical nociception threshold. However, mechanical nociception of rats treated with DMSO + saline started to resolved from 1 hour (161.7 ± 13.7 g) and the pain threshold was maintained until 24 hours (154.7 ± 16.0 g). As expected, injection of 2% λ-carrageenan results in decreasing of mechanical nociception threshold at all-time points until 24 hours. Pain threshold of the rats injected with carrageenan decrease almost 50% at 24 hours (81.24 ± 10.4 g) when compared to the basal reading (147.2 ± 13.1 g). Interestingly, in this study, we have demonstrated that rats that received 50mg/ml of Fructus viticis crude extract prior to carrageenan injection has showed a significant ($p<0.05$ & $p<0.01$) increased of mechanical nociception threshold (resolution of pain) when compared to rats injected with DMSO+Carrageenan at 1 hour (Extract+Carr: 123.7 ± 9.1 g vs DMSO+Carr: 63.6 ± 8.7 g) to 6 hours (Extract+Carr: 97.0 ± 9.7 g vs DMSO+Carr: 37.6 ± 3.9 g). However, after 8 hours to 24 hours there was no significant analgesic effect of the extract when compared to the DMSO+carrageenan group as shown in Figure 4.4. Fructus viticis crude extract that was used as treatment did not cause any significant increase in pain as there is no significant reduction of mechanical nociception threshold at all-time point when compared to control (DMSO+vehicle) as shown in Figure 4.4.

Nitric oxide is one of the inflammatory mediators that will sensitise nociceptor neuron thus will results in pain. Since LNMMA is nitric oxide synthase inhibitor, the rats treated with LMNNA are expected to reduce pain thus increase the pain threshold. However, there is no significant difference of pain threshold of rats in LNMMA + carrageenan group when compared with DMSO + carrageenan group.

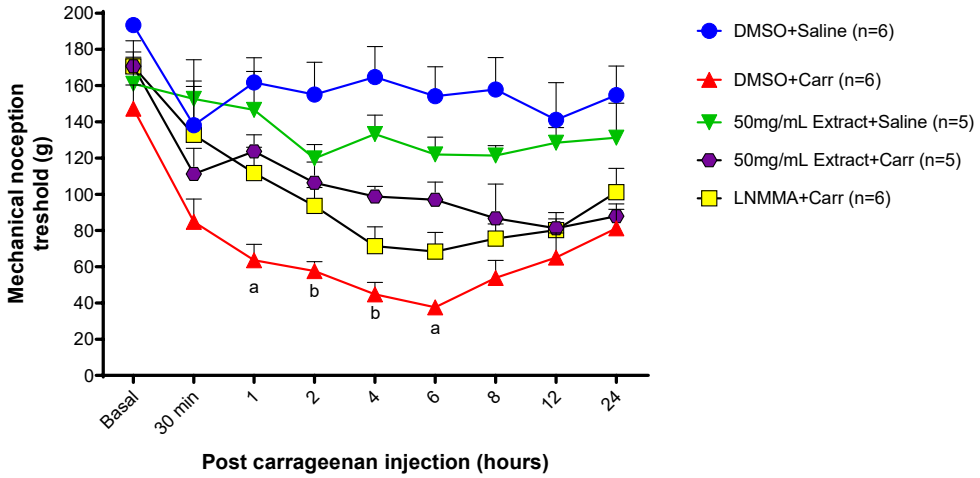

Figure 4.4 Comparison of mean mechanical nociception threshold (g) of rats treated with different treatment at different time interval The mechanical nociception threshold were measured using Ugo Basile Analgesy-Meter of Randal-Selitto. Fructus viticis extracts produces analgesic effect by delaying acute pain at certain time points which are from 1 to 6 hours $^{a}P<0.01$ & $^{b}P<0.05$ when compared with DMSO + carrageenan. .

4.4 Blood pressure

Systolic blood pressure of the rats at different time intervals was ranged between (80-180 mmHg). The result of systolic blood pressure shows that there were no significant different between all groups of the animals at all-time interval (Figure 4.5). These indicate that there were no systemic and centralize pain experienced by the rats. The rats only experienced peripheral and localize acute pain as a result of 2% λ-carrageenan.

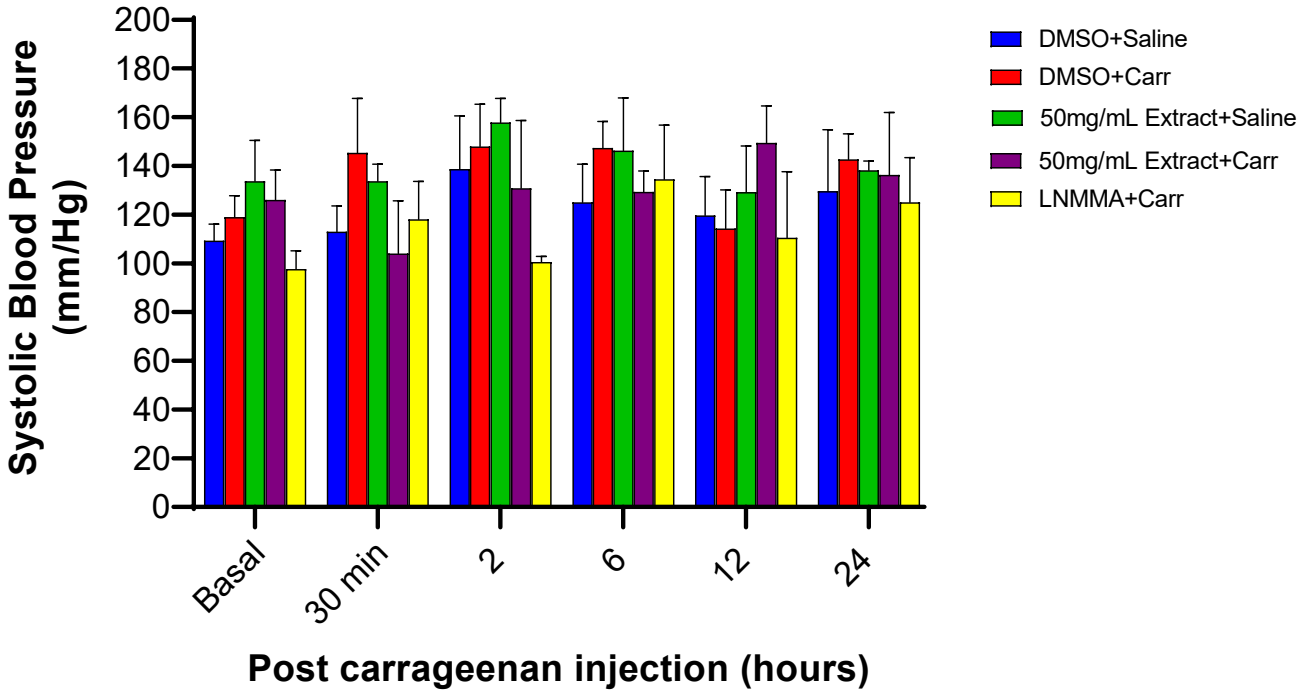

Figure 4.5 Comparison of mean systolic blood pressure at different time intervals. Blood pressure of all rats were measured using the Mouse Rat Blood Pressure (MRBP) non-invasive tail cuff method. No significant different in blood pressure measurement in comparison between all treatments thus indicates that the animals did not experienced severe pain at all-time points.

4.5 Full blood count (FBC)

As shown in Figure 4.6, there were no significant different in total red blood cells, hemoglobin, hematocrit-packed cell volume (HPCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW) and platelet when compared between different groups of treatment.

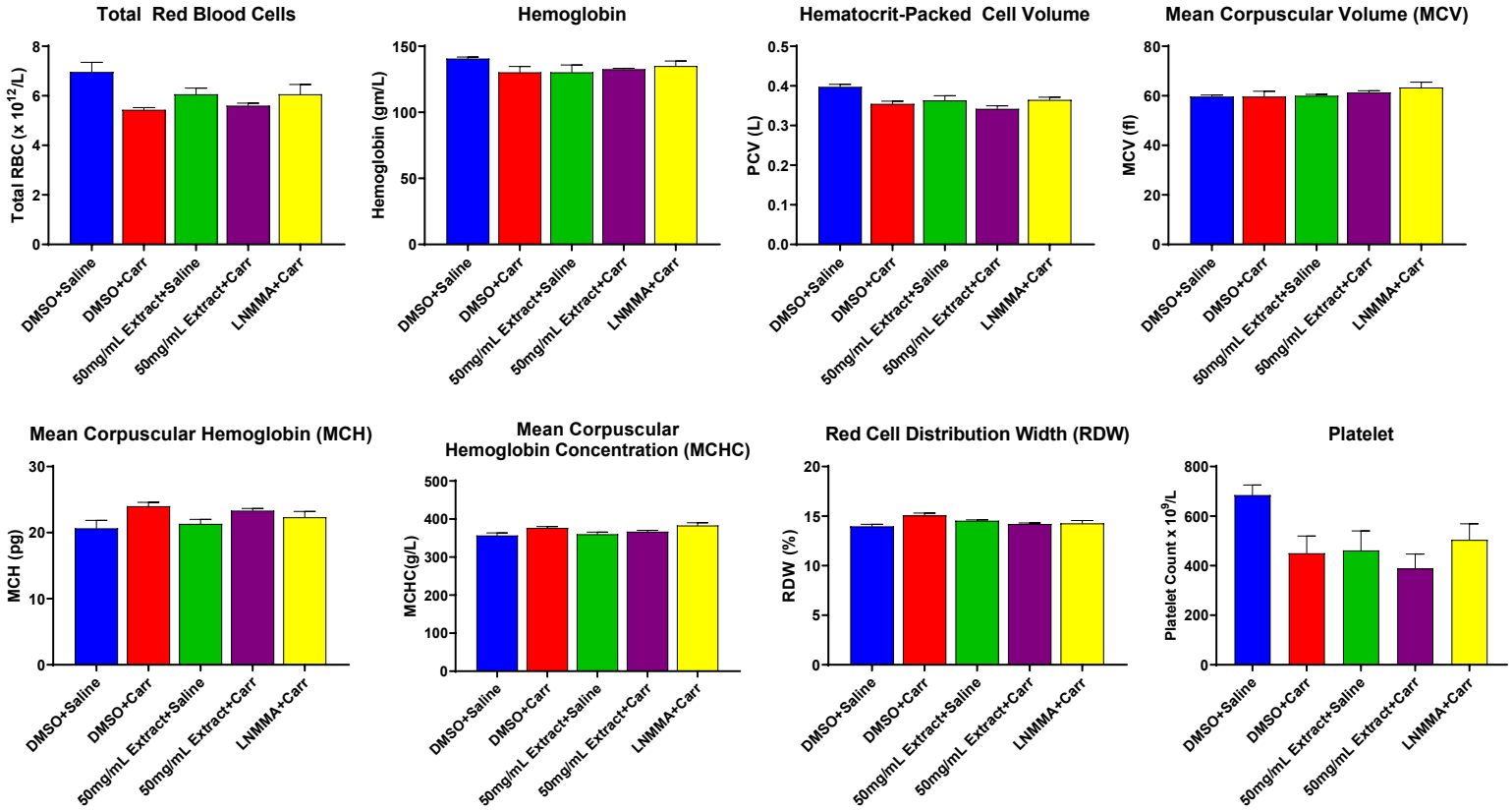

Figure 4.6 There are no significant different in total RBC, hemoglobin, HPCV, MCV, MCH, MCHC, RDW and platelet between different group of treatments.

4.5.1 Effects of the treatment on total white blood cells

Full Blood Count (FBC) analysis revealed that DMSO+carrageenan injection in the paw had cause a significant ($P<0.05$) elevation of white blood cells (WBC) (8.4 ± 1.0 %) when compared to control rats (DMSO+Saline) (3.7 +0.4 %). However, there was no significant reduction of WBC in the rats treated with 50mg/mL extract + carrageenan (8.2 ± 0.8 %; $p<0.05$) when compared to DMSO+Carrageenan and the WBC counts was significantly higher when compared to control group. No significant different of WBC in the extract+saline and LNMMA+Carrageenan when compared to control group as shown in Figure 4.7.

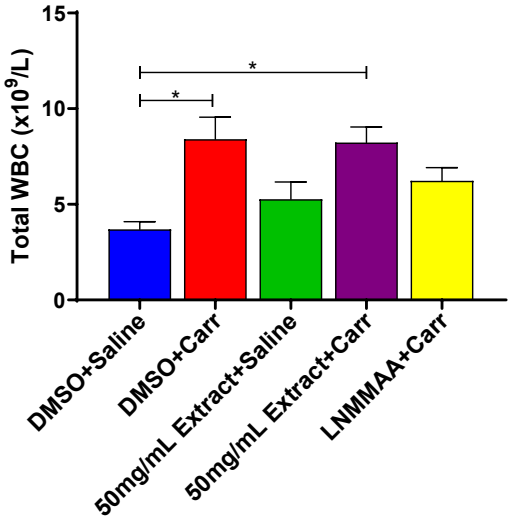

Figure 4.7 The effect of treatment and carrageenan on total white blood count. Carrageenan has caused a significant increase of the total white blood count when compared to DMSO + Saline treated rats. Data are presented as mean values of WBC count + SEM *$P<0.05$ ($n=3$).

4.5.2 Effects of the treatment on neutrophil

Statistical analysis on neutrophils count in blood showed no significant difference among groups at 24 hours post injection. However, result showed elevation in percentage of neutrophils in blood of carrageenan-induced rats when compared to control group. neutrophils count seems to be higher in DMSO + carrageenan (48 ± 15.1 %), 50 mg/mL extract + carrageenan (43 ± 13.8 %) and LNMMA + carrageenan (41 ± 6.7 %) treated rats compared to DMSO + saline (12.3% ± 2.2 %) as shown in Figure 4.8.

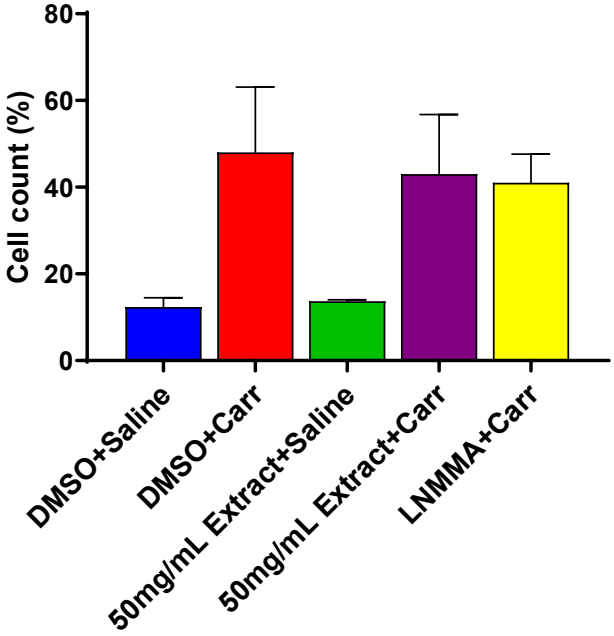

Figure 4.8 The effect of treatment and carrageenan on neutrophils. There is no significant difference among groups. However carrageenan-induced rats show elevation in percentage of neutrophils in blood when compared to DMSO + Saline treated rats.

4.5.3 Effects of the treatment on monocyte

Full Blood Count (FBC) analysis revealed that DMSO+carrageenan injection in the paw had cause a significant ($P<0.0001$) elevation of monocytes (10.0 ± 0.6 %) when compared to control rats (DMSO+Saline) (2.3 +0.3 %). Interestingly, analysis on the monocytes count revealed that there was significant ($p<0.01$) reduction of

monocytes counts between the treatment, Frutus viticis extract + carrageenan (4.7 ± 0.7 %) and carrageenan group, DMSO + carrageenan (10.0 ± 0.6 %) as shown in Figure 4.9. In addition, Fructus viticis extract did not cause elevation of monocytes counts in FBC analysis as there was no significant alteration of monocytes in the Extract+saline (2.67 ± 0.89) when compared to DMSO+Saline (2.33 ± 0.33) . Furthermore, LNMMA + carrageenan treated rats showed a significant ($p<0.0001$) reduction of monocytes count (2.3 ± 0.8 %) when compared to carrageenan group.

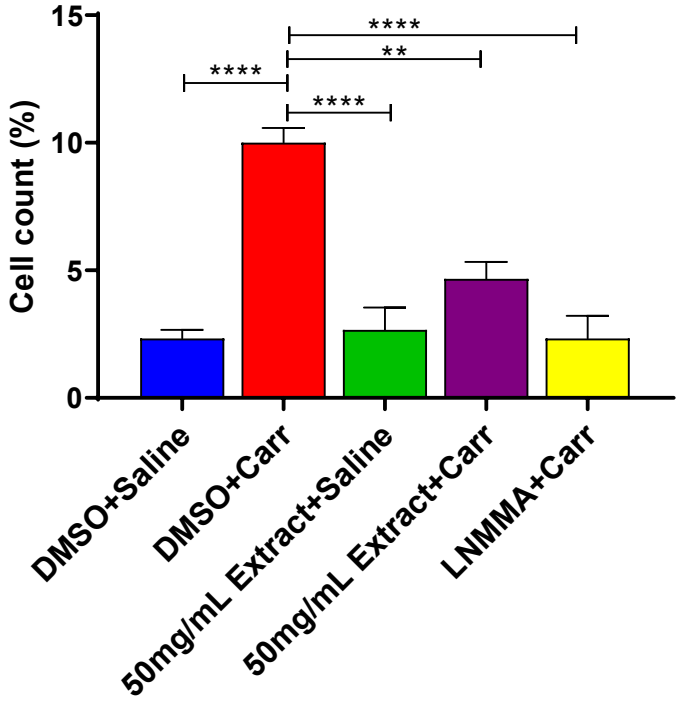

Figure 4.9 Effect of treatments on the infiltration of monocytes at 24 hours post carrageenan or saline injection. DMSO + carrageenan treated rats thus results in significant increases of monocytes count when compared to other group. **P<0.01 & ****P<0.0001 (n=3).

4.5.4 Effects of the treatment on lymphocyte, eosinophils and basophils

As shown in Figure 4.10, there was no significant difference in the infiltration of lymphocytes and eosinophils at 24 hours post carrageenan or saline injection despite of different in treatments. Moreover, there was no eosinophils infiltration in DMSO + carrageenan and LNMMA + carrageenan treated rats while eosinophils percentages in other group were ranged between 1-3 % (1.6 ± 0.3 %). On the other hand, average lymphocytes counts in all animals were between 44-87 % (69.8 ± 3.2 %). From the FBC analyses, no basophils were detected from the whole blood collected at 24 hours post saline or carrageenan injection.

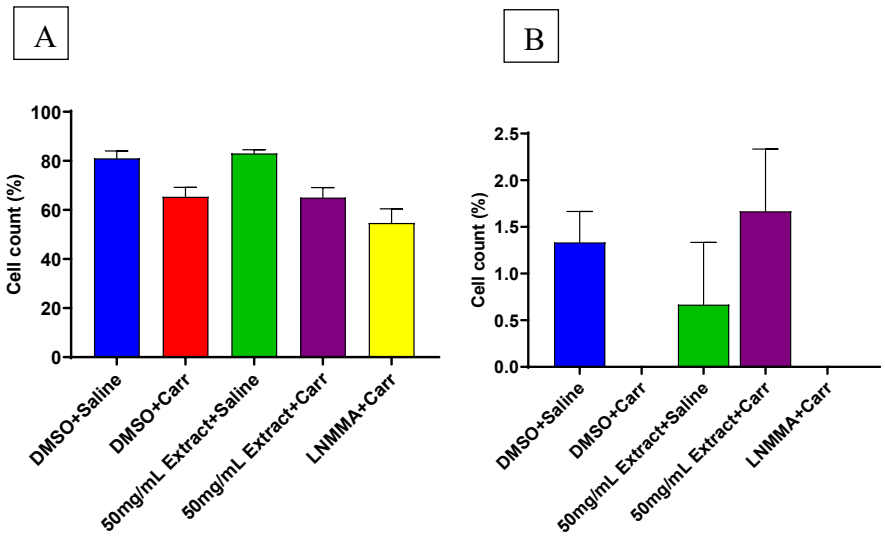

Figure 4.10 Effect of treatments on the infiltration of lymphocytes (A) and eosinophils (B) from whole blood at 24 hours post carrageenan or saline injection. No significant effect of treatments on the lymphocytes and eosinophils count in the whole blood.

4.6 DPPH Antioxidant Activity of Fructus viticis methanolic extract

The antioxidant activity of the Fructus viticis methanolic extract was measured by the ability to scavenge DPPH free radicals and was compared with the standard BHT. As expected the BHT showed excellent radical scavenging activities with maximum inhibition (81.40%) while maximum inhibition for the extract was

(78.82%) at 1 mg/mL. IC_{50} for BHT and extract was 0.145 mg/mL and 0.365 mg/mL respectively.

Table 4.1 DPPH radical scavenging activity of Fructus viticis methanolic extracts with concentrations ranging from (0-1 mg/mL). BHT was used as a positive control.

Concentrations (mg/mL)	Mean value ± S.D	Extract IC_{50} values (mg/mL)	BHT (Mean value ± S.D)	BHT IC_{50} values (mg/mL)
0	0		0	
0.0156	9.26 ± 1.65		8.524 ± 0.00	
0.0312	11.15 ± 1.17		16.62 ± 0.00	
0.0625	16.06 ± 1.37	0.365	32.14 ± 0.00	0.145
0.125	22.91 ± 1.04		46.76 ± 0.00	
0.25	35.23 ± 0.19		62.08 ± 0.00	
0.5	60.95 ± 4.90		76.51 ± 0.00	
1	78.82 ± 7.43		81.40 ± 0.00	

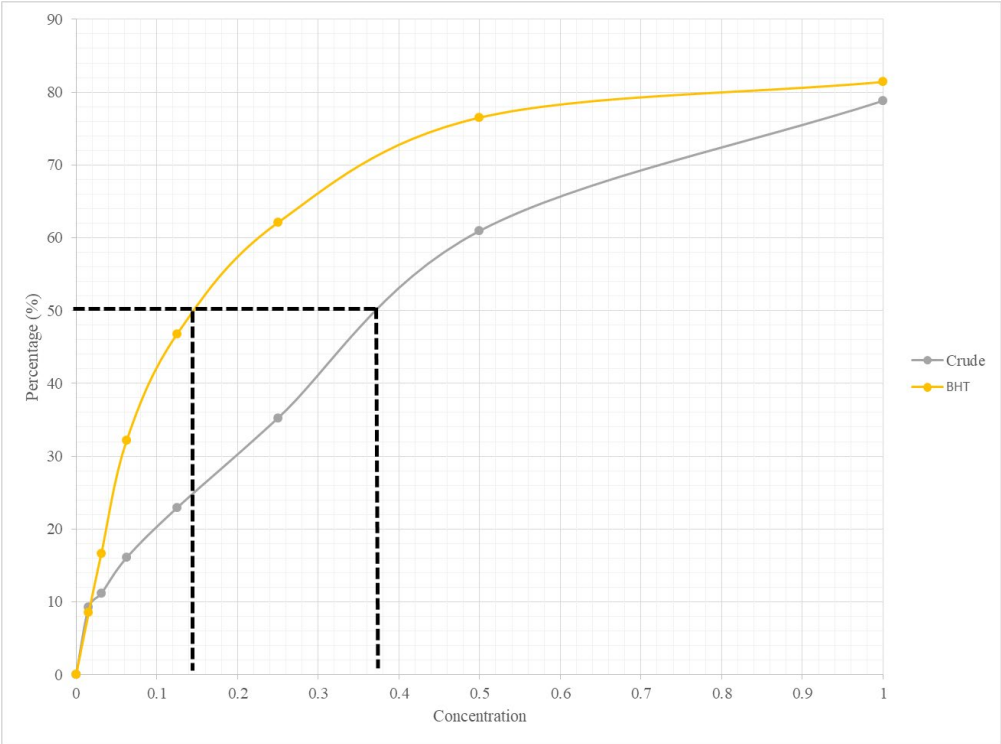

Figure 4.11 Comparison of antioxidant activity of Fructus viticis methanolic crude extracts at different concentration. The antioxidant assay was done by using DPPH radical scavenging assay. Determination of 50% inhibition concentration (IC_{50}) for BHT is 0.145 mg/mL and extract is 0.365 mg/mL.

4.7 Expected Results

4.7.1 Effects of the Treatment on Nitric Oxide (NO) Concentration in Paw Tissues Collected at 24 Hours Post Saline or Carrageenan Injection

NO is one of the important mediator in acute and chronic inflammation is generated via the oxidation of the terminal guanidino nitrogen atom of L-arginine by the enzyme, nitric oxide synthase (NOS). In our study, we expected that carrageenan injection cause elevation of nitric oxide production in the hind paw. Many reports have showed that injection with 1-3% of carrageenan has elevated the NO production in hind paws. Study by Mizokami *et al*., (2016) has shown that intraplantar injection of carrageenan on Male Swiss mice was able to induce nitric oxide (NO) production in the peritoneal cavity (Figure 4.12). Study by Boschi et al., (2008) showed that carrageenan increase the concentration of nitric oxide (NO) in the pleural cavity when compared to control group (Cg: 86.02 3.86 nmol, P ¼ 0.001) vs saline group (49.78 5.63 nmol)) (Figure 4.13).

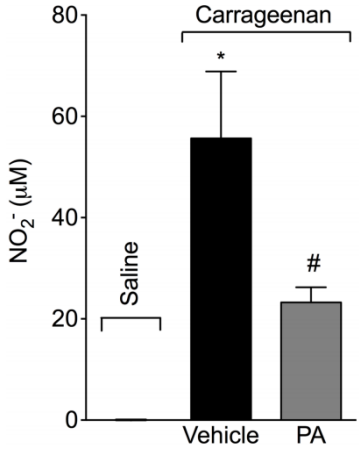

Figure 4.12 Nitrite production in peritoneal exudates was determined 3 hours after carrageenan injection (Mizokami *et al*., 2016)

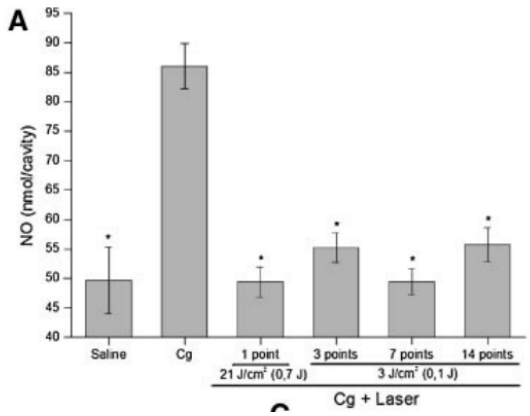

Figure 4.13 NO concentration in carrageenan and control group. Results are expressed as means SEM of eight animals. *P<0.05 (Boschi *et al*., 2008).

Study by Salvemini,*et al*., 1996 has shown that intraplantar injection of carrageenan in male Sprague-Dawley rats increased in paw volume with an elevated production of NO2-/N03- in the paw exudates (Figure 4.14). This increase in N02-/NO3- was observed within 30 min (from 0.5 + 0.05 nmol/paw to 16.2 +4 nmol/paw, n = 6), remained constant for the subsequent 3 h and then increased further at 6 and 10 h following carrageenan administration (Figure 4.14 a and b). In addition, based on this study, L-NMMA (300 mg/kg, n = 5) inhibited the N02-/NO3- production in paw exudate at both 3 and 10 h after carrageenan administration (Figure 4.14 a and b). Therefore, we expect LNMMA will inhibit the production of NO 24 hours after injection with carrageenan. In addition, our treatment (50 mg/mL extracts + carrageenan) should inhibit/reduce the NO production at 24 hours post carrageenan injection since the treatment was found to delay the development of oedema.

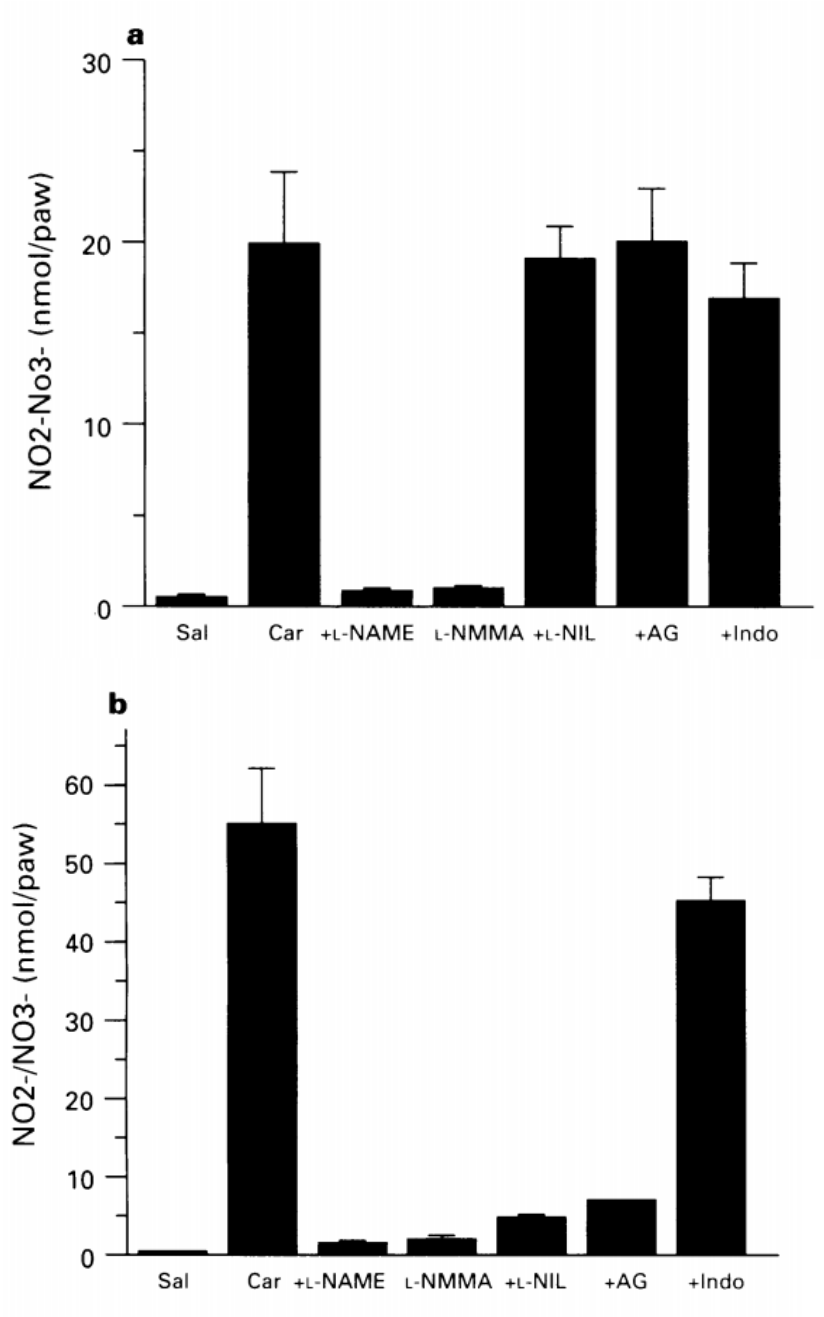

Figure 4.14 Effects of carrageenan on NO2-/NO3- production at 3 (a) and 10 (b) h after carrageenan administration. The non-selective NOS inhibitors (LNMMA) inhibited NO_2^-/NO_3^- production at 3 and l0h (a and b), Each point is the mean+s.e.mean for n = 6. (Salvemini,*et al*., 1996).

4.7.2 Quantification of Inflammatory Cells in H&E Stained Paw Tissue

FBC analysis has revealed that carrageenan significantly increased monocytes count percentage when compared with DMSO + carrageenan treated rats. Carrageenan is

known to activate macrophages that will drive the local inflammatory reaction, and inflammatory cell infiltration. Therefore, histology analysis of paw tissue of carrageenan treated rats are expected to results in massive infiltrations of immune cells especially monocytes. We expect the DMSO + carrageenan group will results in more inflammatory cells infiltration compared to DMSO + saline group. Histological study by Buisseret *et al*., 2019 to assess immune cell infiltration of the paw was established at 6 and 24 h following carrageenan injection and revealed that carrageenan can induced a large infiltration of inflammatory cells in the paw, as seen in representative pictures (Figure 4.15 A and C). Consistently, a higher score was obtained in this group compared to the vehicle group for both time points. Study by Sadeghi *et al*., 2013 also suggests that carrageenan able to cause tissue changes, PMN infiltration, and swelling (Figure 4.16).

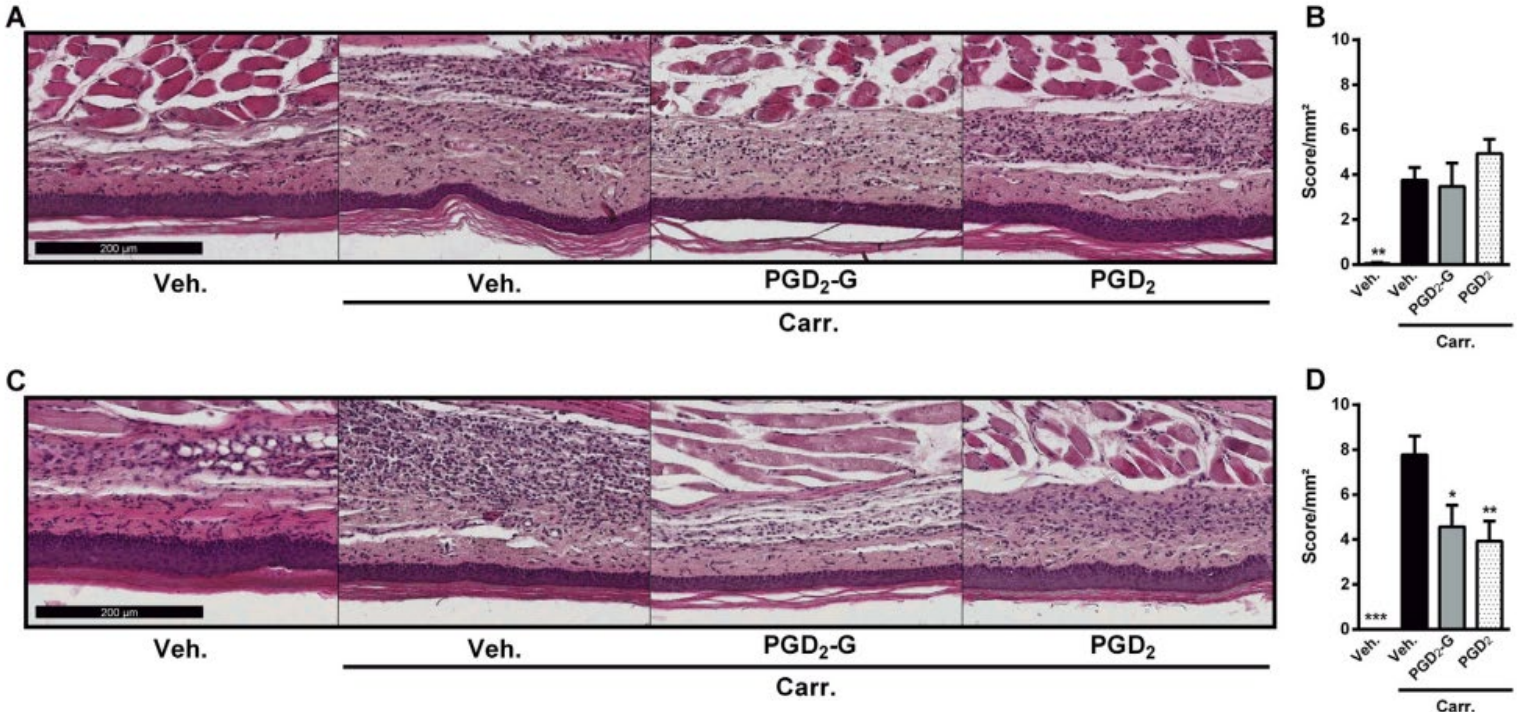

Figure 4.15 Mice were injected with 25 µL of carrageenan solution or saline vehicle into the plantar side of the right hindpaw. Mice were sacrificed at 6 h (A and B) or 24 h (C and D) after carrageenan injection. Representative pictures of hematoxylineosin stained sections, scale bar at 200 µm. (B and D) Histological scoring was performed at 6 (B) and 24 (D) hours after carrageenan administration. Values are mean ± s.e.m. (n = 8 per group). *P < 0.05, **P < 0.01, ***P < 0.001, compared to vehicle-carrageenan group, using one way ANOVA and Dunnett's post-hoc test (Buisseret *et al*., 2019).

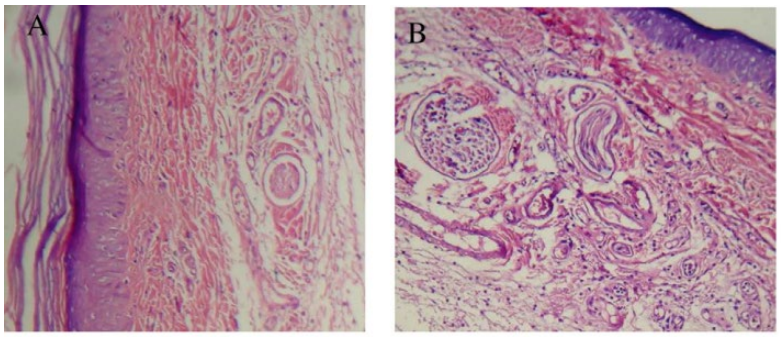

Figure 4.16 Histopathological evaluation of rat paws 4 h after subplantar injection of carrageenan. (A) Appearance of epidermis and dermis in normal rats without any lesion. (B) Subplantar injection of carrageenan induced edema, and migration of leukocytes mainly neutrophils (Sadeghi *et al*., 2013).

Study by Shin et al (2009) showed that carrageenan was able to increase the infiltration of immune cells including neutrophils, monocytes, and lymphocytes. Increase in neutrophils, monocytes, and lymphocytes are more prominent although eosinophils and basophils were slightly increasing following carrageenan injection (Table 4.2).

Table 4.2 Immune cells infiltration after carrageenan injection (Shin *et al* 2009).

Treatment	Vehicle	Carrageenan alone
WBC	0.92 ± 0.29	9.44±3.31*
Neutrophils	0.16 ± 0.06	0.94±0.31*
Eosinophils	0.03 ± 0.02	0.06±0.04
Basophils	0.05 ± 0.04	0.09±0.33
Monocytes	0.07 ± 0.03	0.42±0.15*
Lymphocytes	0.64 ± 0.19	7.82 ± 2.89*

*: Significantly different from vehicle control (P<0.05)

CHAPTER 5

DISCUSSION

In this study, investigation of the effects of Fructus viticis methanolic crude extract on acute inflammatory pain evaluated by carrageenan induced paw oedema was done by assessing four parameters including paw thickness, pain threshold, systolic blood pressure, and full blood count analysis. In addition, antioxidant activity of Fructus viticis methanolic extract was investigated using DPPH radical scavenging assay.

5.1 Fructus viticis effects on carrageenan-induced paw oedema model

Carrageenan is a one of inflammatory agents that is commonly used for induction of acute inflammatory pain by intraplantar injection of animal model to develop paw oedema (Ou *et al*., 2019). Therefore, carrageenan- induced paw oedema model was used in this study as it is a well-defined, widely and frequently used working model of inflammation in the search for new anti-inflammatory drug (Kuedo *et al*., 2016). Furthermore, paw oedema is a convenient method for assessing inflammatory responses to antigenic challenges and irritants (Kuedo *et al*., 2016; Kim *et al*., 2020; Sarkhel, 2016).

The thickness of paw oedema is one of the parameter taken for assessing the development of inflammation. Acute paw oedema are caused by the increased vascular permeability and plasma extravasation which caused accumulation of fluid, leukocytes and mediators at the site of inflammation (Helen *et al*., 2018). Inflammation induced by carrageenan is biphasic (Bao *et al*, 2018; Kim *et al*, 2018)

whereat the early phase (first 2 h after carrageenan injection) is attributed to the release of proinflammatory mediators, such as histamine and serotonin; the late phase (3–5 h after carrageenan injection) is mainly mediated by neutrophil infiltration into the inflammatory site and the production of large amounts of pro-inflammatory mediators such as kinins, prostaglandin, nitric oxide, cyclooxygenase, cytokines such as IL-1β, IL-6, IL-10 and TNF-α, and neutrophil derived free radicals (Moon *et al*., 2018; Ismail *et al*., 2016; Kim *et al*, 2020).

Previous studies have shown that intraplantar injection of carrageenan showed a noticeable difference in gross morphology such as increased redness, hotness, swelling, painful paw tissue oedema (Helen *et al*., 2018; Kim *et al*, 2020; Abd-Allah *et al*., 2018). Similarly, in our study it was demonstrated that injection of 100 µL of 2% λ-carrageenan results in massive oedema (swelling) that characterized by the increase of paw thickness which significantly elevated within 30 minutes after carrageenan injection, reaching peak at 4 h to 8 h before starting to resolve at 24 hours post-carrageenan injection. Paralleled with the study by Ialenti et al., (2017); Abd-Allah et al., (2018); Yuan et al., (2017) where paw oedema started to develop at 0.5 h, where injection of 100 µL of 1% λ-carrageenan of carrageenan to the rat hind paw caused an oedema peaking between 3 and 4 hours. The peak time of eodema development is slightly different from our study because of the different carrageenan concentration used where in our study we used 2% (w/v) of λ-carrageenan instead of 1% (w/v).

Moreover, gross observation of rat hind paw treated with Fructus viticis extract showed less cardinal sign of inflammation including redness, heat, swelling and pain

compared to control (DMSO + Carrageenan). Our study has revealed that animal treated with Fructus viticis extract showed significant delay in the development of oedema at 4 h to 6 h post carrageenan injection when compared to control group (DMSO + Carrageenan). The ability of the extract to resolve the inflammation might be due to the bioactive compound in the crude extract that inhibit the known classical inflammatory pathway such as the Toll like receptor-4 (TLR4) mediated Nuclear factor kappa B (NF-κB) activation that control the regulation of proinflamamtory mediators. Study by Liou and Huang., (2017) showed that casticin, a bioactive compound in Fructus viticis could suppress the inflammatory effect by blocking the NF-κB and MAPK pathways in TLR4 ligand LPS-induced RAW264.7 macrophage cells and decreases the levels of eotaxin and reduces eosinophil migration in IL-1β-stimulated A549 human lung epithelial cells. Moreover, casticin may serve as a potential anti-inflammatory and anti-nociception agent when it significantly improved cell viability in chondrocytes exposed to IL-1β by inhibiting IL-1β-induced NO and PGE2 production, iNOS and COX-2 expression in human osteoarthritis chondrocytes and suppressed the levels of TNF-α and IL-6, as well as decreased production of MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 in IL-1β-stimulated chondrocytes. Study by Choi *et al*., (2010) on the effect of *V. rotundifolia* on the production of NO in IFN-gamma and LPS-stimulated mouse peritoneal macrophages showing that *V. rotundifolia* suppressed nitric oxide (NO) production, iNOS and COX-2 expression dose-dependently through suppression of NF-κB activation without notable cytotoxicity thus may serve as a potential anti-inflammatory and anti-nociception agent.

5.2 Systolic blood pressure measurement on carrageenan-induced inflammation model

Carrageenan triggers the releasing of various cytokines that play critical role in the oedema formation, mechanical allodynia, neutrophil migration, and in pain hypersensitivity (Annamalai and Thangam, 2017). Therefore it was important to ensure that the animals did not experience intense systemic pain as the carrageenan are expected to only cause localized acute pain. It is known that pain can give rise to the blood pressure and hypertension as well as heart rate (Sacco *et al.*, 2013). Over activity of the sympathetic nervous system (SNS) often leads to cardiovascular disease such as hypertension as well as increasing heart rate (Wang *et al.*, 2017).

Therefore blood pressure was used as indicator in pain development of animal models to ensure animals do not experience excruciating pain that can interrupt the animal's welfare. Systolic blood pressure (SBP) of all animals that was measured by using tail-cuffed method has shown that there is no significant different blood pressure when compared statistically between groups. Therefore, it can be concludes that the animals did not experienced systemic pain since the carrageenan only can induce acute inflammatory and localized pain at the site of injection.

5.3 Fructus viticis as analgesic agent in acute pain

The peripheral sensitization can be triggered by NF-κB-related pro-inflammatory mediators, including the cytokines TNF-α and IL-1β, as well as ROS, such as the superoxide anion radical (Kuedo *et al*., 2016). Carrageenan is found to induce inflammation and pain by direct binding to and activation of the TLR4 and NF-κB pathway thus induces oxidative stress (J.M. McKim Jr. *et al*., 2016; David *et al*., 2020). Therefore, carrageenan model is frequently used to produce unilateral painful

inflammation (Harris-Bozer and Peng, 2016; Rock et al., 2018). Carrageenan injection induced the expression of some inflammatory markers such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, macrophage inflammatory protein (MIP)1α, COX2 and the macrophage activation marker CD11c thus causing peripheral hyperalgesia (Buisseret *et al*., 2019).

Study by Lauro *et al*., (2016) has shown that 1% intraplantar injection of carrageenan in rats hind paw produces a time dependent development of thermal hyperalgesia (pain sensation) which peaks within 2–3 h and lasts for another 6 h. In our study, rats injected with vehicle + carrageenan have significantly reduced mechanical nociception threshold from 1–6 h post carrageenan injection indicating increase in pain sensation. Interestingly, our study has revealed that rats that receiving 50mg/ml of Fructus viticis crude extract 30 minutes prior to carrageenan injection has showed a significant increase of mechanical nociception threshold (resolution of pain) when compared to rats injected with DMSO+Carrageenan at 1 hour to 6 hours. Study has found that casticin, a bioactive compound of *V. rotundifolia* had significant anti-nociceptive using acetic acid writhing test and anti-inflammatory effect on acute inflammation by xylene-induced ear edema (Ramezani *et al*., 2010). Moreover, methanolic extract of the fruits of *V. rotundifolia* showed the inhibitory effect on the NO production in RAW264.7 cells (Lee *et al*., 2013). In addition, diterpenoids that were isolated from Fructus viticis has significantly inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW264.7 cells thus involves in anti-inflammatory activities (Yao *et al*., 2016). Instead of casticin, other compound present in *V.rotundifolia* such as aucubin which is another iridoids that can also be found in *V. rotundifolia* and this compound

possess anti-inflammatory activity when study revealed aucubin able to inhibit TNF-α production in RAW 264.7 cells (Kyoung and Chang, 2004).

5.4 Full Blood Count Analyses

Earlier in the study we have shown that carrageenan has caused massive oedema probably due to the increase of immune cells infiltration. It has been reported that carrageenan can induce inflammatory activities by increasing macrophage phagocytosis, antibody production, lymphocyte proliferation, natural killer (NK) cell and NKT cell activity, pro-inflammatory cytokine secretion (Li *et al*., 2017). Our study has shown carrageenan has caused massive elevation of white blood cells when compared to control rats. However, Fructus viticis extract did not significantly reduced WBC and the WBC count was significantly higher when compared to control group. Our study has shown that neutrophils count in blood shows no significant difference among groups at 24 hours post injection. However, result shows the percentage of neutrophils in blood of carrageenan-induced rats is the highest when compared to other groups. Caiazzo *et al*., (2016) revealed that neutrophils dominated the early phase (4 h) of the reaction and were replaced by monocytes at 72 h probably the reasons in our study there was no significant difference in neutrophils counts since the blood was taken 24 h after carrageenan injection.

TNF-α, IL-1β, and IL-2 are pro-inflammatory cytokines produced by immune cells macrophages and monocytes in response to inflammation and cellular injury and causing pain (Zhang *et al*., 2020). Earlier study has shown that animals treated with carrageenan have developed intense hyperalgesia when they have lowered

mechanical nociception threshold, probably due to high infiltration of monocytes. Full Blood Count (FBC) analysis revealed that rats injected with carrageenan showed significant elevation of monocytes when compared to control rats. Interestingly, analysis on the monocytes count revealed that there was significant reduction of monocytes counts between the treatment, Fructus viticis extract and carrageenan group. Previous study has found that casticin isolated from Fructus viticis could suppress the inflammatory effect by blocking the NF-κB and MAPK pathways in LPS-induced RAW264.7 macrophage cells and decreases the levels of eotaxin and reduces eosinophil migration in IL-1β-stimulated A549 human lung epithelial cells (Liou *et al*., 2017). Furthermore, LNMMA + carrageenan treated rats also showed a significant reduction of monocytes count when compared to carrageenan group.

As mentioned at the beginning, we have demonstrated that in FBC analysis carrageenan caused massive immune cells infiltration in the blood circulation. Therefore, histological analysis is required to further investigate the mechanism on how Fructus viticis extract exhibits its anti-inflammatory and analgesic effect particularly on the infiltration of immune cells in the paw tissues. However, due to COVID-19 pandemic and Malaysia Control Order (MCO), the fixed paw tissues are unable to be processed for histopathological analyses. Histopathological evaluation of the paw tissue of carrageenan-injected mice revealed epithelial hyperplasia, infiltration of inflammatory cell, and subepidermal oedema (Zhang *et al*., 2020). According to Jisha *et al*., (2019), carrageenan can cause manifestation of inflammatory cell infiltration, proliferated epithelium, proliferated collagen, epidermal oedema. Histopathological analysis of paw tissue has shown that paw tissue of the normal rats showed no signs of inflammation with normal keratin, sub

epidermal layer and sub cutaneous layer while rats treated with carrageenan shows massive influx of inflammatory cell infiltration, proliferated collagen, hyper keratotic skin, sub epidermal oedema, after 5 h after carrageenan injection (Helen et al., 2018; Jisha et al., 2019).

5.2 Fructus viticis effects on carrageenan-induced paw oedema model

Our study has revealed that Fructus viticis crude extract possesses high antioxidant activity when compared with BHT. Study has shown that methanolic extract of the twigs of *V. rotundifolia* possesses potent antioxidant activity when measuring the radical scavenging effect on DPPH (1,1-diphenyl- 2-picrylhydrazyl) when 2 flavanoids, orientin and a quinic acid derivative, 3,4-di-O-caffeoylquinic acid showed the significant antioxidative effects Lee *et al*., 2018, Yao *et al*., 2016, Kim, 2009). Furthermore, ferruginol, an abietane-type diterpenoid isolated from *V. rotundifolia* showed higher antioxidant activity than 3-tert-butyl-4-hydroxyansiole (BHA) using the ferric thiocyanate method (Yao *et al*., 2016). Study by Domingues et al., (2019) has shown that increases antioxidant presence in the intra-abdominal areas such as omental fat and ameliorates a prevalent metabolic syndrome complication such as fatty liver disease by promoting browning of white fat and more importantly reducing systemic inflammation. Bognar *et al*., (2013) revealed that high antioxidant compound augments LPS-induced Akt activation and MKP-1 expression and attenuates mitochondrial destabilization, ROS production and activation of PARP as well as MAPKs resulting eventually in diminished activation of NFκB thus significantly contributes to the anti-inflammatory effects. The anti-inflammatory action might be due to the overexpression of antioxidant enzymatic

systems leads to excess reducing equivalents that can deplete ROS, driving the cells to reduce stress eventually reduce inflammation and pain (Pérez-Torres *et al*., 2017).

CHAPTER 6

CONCLUSION

As conclusion, methanolic extract of the *Vitex rotundifolia* fruits known as Fructus viticis exhibits anti-inflammatory effects by delaying the development of carrageenan-induced paw oedema at 4 h to 6 h which subsequently produces analgesic effect at certain period. Moreover, the ability of Fructus viticis extract to reduce the paw oedema as well as pain is suggested to be associated with the potential of the extract to reduce the infiltration of inflammatory cells which specifically the monocytes/macrophages into the hind paw. Interestingly, all treatment did not have significant effects on other type of immune cells such as lymphocytes, eosinophils and basophils which further suggest that the extract did not affect the innate immunity and allergic reaction. Moreover, anti-inflammatory and analgesic effects of Fructus viticis extract might be the direct consequences of antioxidant activity of Fructus viticis. Overall, the fruit of *V.rotundifolia* has a potential to be developed into a novel antiinflammatory and analgesic drugs and other pharmacological products in future.

6.1 Limitations

Some of the many limitations that we have encountered throughout the study were assessing the pain behaviour of the rats using Randall-Selitto test. A few rats exhibited uncomfortable behaviour and hypersensitive even with prior sufficient acclimatization and handling. This occurrence indirectly delayed the time as we required the rats to be as calm as possible to yield a consistent and reproducible data. Moreover, paw oedema was measured by using a Digital Vernier Caliper which

measures the paw thickness between the plantar and dorsal of the paw. This technique only measures certain area of the paw which can be bias among different experimenter. Another method used to determine the types of inflammatory cells infiltrated was less precise as it measures inflammatory cells in the systemic circulation using full blood count analyses, and not quantified from the paw tissues directly. Moreover, the blood was taken only after 24 h post carrageenan injection. This data may not precisely represent the present of specific immune cells in blood, especially at the early phase of carrageenan induction. Furthermore, some part of our study such as histopathological analyses and determination of inflammatory mediator nitric oxide (NO) cannot be conducted because of Covid-19 pandemic and Malaysia Control Order (MCO).

6.2 Recommendation

The development of paw oedema and the effects of treatment on the paw can be measured accurately by using other alternative apparatus such as plesthymometer that measure the total volume of the whole paw. Von Frey Filament and Incapacitance machine are another alternative in assessing pain behaviour as this method does not require the experimenter to hold the animals which can reduce the stress of the animal. This will yield to a consistent and reproducible data as the animals are calmer. In this study, it is vital to determine the specific inflammatory cells in the paw tissue; therefore the most suitable method is by applying immunohistochemistry onto the paw tissue. Moreover, the experimental time can be reduce from 24 h to 12 h as our study has shown that carrageenan induce inflammation and pain at 1-6 h.

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APPENDICES

Appendix A

Animal Ethics Approval

Jawatankuasa Penjagaan dan Penggunaan Haiwan
Institusi USM (JKPPH USM)
USM Institutional Animal Care and Use Committee
(USM IACUC)

Bahagian Penyelidikan & Inovasi (R&I)
Kampus Kesihatan,
Universiti Sains Malaysia
16150, Kubang Kerian, Kelantan

Tel: 09-767 3000 samb. 2364 / 2352
W: www.research.usm.my

13th May 2019

Dr. Wan Amir Nizam Wan Ahmad
School of Health Sciences
Universiti Sains Malaysia
16150 Kubang Kerian
Kelantan

Dear Dr.,

Animal Ethics Approval

Project title (983) : Elucidation of Anti-Inflammatory Mechanisms of Vitexicarpin on Immune Cells Activation *In Vivo* and *In Vitro*

The USM Institutional Animal Care and Use Committee (USM IACUC) has approved the above research project.

No. of Animal Ethics Approval: USM/IACUC/2019/(117)(983)

Title	: Elucidation of Anti-Inflammatory Mechanisms of Vitexicarpin on Immune Cells Activation *In Vivo* and *In Vitro*
Source of Animals	: Animal Research and Service Centre (ARASC), Health Campus
Location of Animals	: Animal Research and Service Centre (ARASC), Health Campus
Duration	: 13th May 2019 – 13th May 2021
Number of Samples	: 106 Sprague-Dawley Rats (Male)
Name of Principal Investigator	: Dr. Wan Amir Nizam Wan Ahmad
Name of Co-Investigator	: Dr. Suvik Assaw : Miss Nurul Laili binti Rosli

Appendix B

Sodium Pentobarbital Calculation

From 60 mg/kg: let's say rat's weight=250g (0.25kg)

$$\frac{60\ mg}{1\ kg} = \frac{x}{0.25}$$

$$x = \frac{60 \times 0.25}{1}$$

$$x = 15\ mg$$

$$\frac{60\ mg}{1\ mL} = \frac{15\ mg}{x}$$

$$x = \frac{15}{60}$$

$$x = 0.25\ mL$$

From here, the volume of dose injected to the rats is determined based on the rat's body weight