THE EFFECTS OF MITRAGYNINE FROM *MITRAGYNA SPECIOSA* ON THE mRNA EXPRESSION OF COX-1 AND COX-2 IN LIPOPOLYSACCHARIDE-STIMULATED RAW264.7 MACROPHAGE CELLS

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by

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Zulkhurnain bin Utar

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
Ab	Antibody
AcOH	Acetic acid
ALT	Alanine transaminase
APS	Ammonium persulfate
ATCC	American Type Culture Collection
AST	Aspartate aminotransferase
bp	base pair
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer-binding protein
CAM-1	Cell adhesion molecule-1
CAM-2	Cell adhesion molecule-2
c-AMP	Cyclic adenosine monophosphate
c-Jun	Member of AP-1 family of transcription factors
CO_2	Carbon dioxide
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
cm	centimeter
C_{max}	The peak plasma concentration of a drug after administration
CL	Clearance
CNS	Central nervous system
CREB	cAMP response element-binding protein
C_t	Threshold cycle
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	double stranded DNA
e.g.	Example guide
EDTA	Ethylene diaminetetraacetic acid
ESI	Electronspray ionization
FBS	Fetal bovine serum

FST	Forced- swimming test
g	gram
h	hour
HPA	hypothalamic pituitary adrenal axis
HPLC-UV	High pressure liquid chromatography with ultraviolet
HRP	Horseradish peroxidase
IC_{50}	Half maximal inhibitory concentration
IgG	Immunoglobulin G
LC_{50}	Medial lethal dose
LCMS-QTOF	Liquid chromatography mass spectrometry quadrupole Time-of-Flight
LPS	Lipopolysaccharide
LTB4	Leukotriene B_4
M	Molar
MeOH	Methanol
mg/kg	Milligram per kilogram
mRNA	messenger ribonucleic acid
NF-κB	Nuclear factor-kappa-B
NSAIDs	Non-steroidal anti-inflammatory drugs
p53	Tumor suppressor protein 53
PAF	Platelets activating factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandins
PGE_2	Prostaglandin E_2
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
$t_{½}$	The time required for the concentration of the drug to reach half of its original value
T_{max}	Time to reach C_{max}
TEMED	N, N, N', N'-tetramethylethylenediamine
TGI	Total growth inhibition
TST	Tail suspension test

LIST OF SYMBOLS

%	Percentage
°C	Degree Celcius
µl	Micro liter
µg	Microgram
β	Beta
Δ	Delta

KESAN MITRAGININ DARIPADA M*ITRAGYNA SPECIOSA KE* ATAS EKSPRESI mRNA BAGI COX-1 DAN COX-2 MENGGUNAKAN SEL MAKROFAJ RAW264.7 TERAWAT LIPOPOLISAKARIDA

ABSTRAK

Mitragyna speciosa Korth (Rubiaceae) merupakan tumbuhan ubatan yang digunakan secara tradisional untuk merawat pelbagai jenis penyakit terutamanya di Thailand dan Malaysia. Maklumat kajian bagi anti-inflamasi dan analgesik menggunakan ekstrak telah banyak didokumenkan. Dalam kajian ini, mekanisme sel bahan bioaktif utama mitraginin telah dikaji kesannya terhadap anti-inflamasi. Kesan mitraginin terhadap ekspresi mRNA dan ekspresi protein bagi COX-1 dan COX-2 serta penghasilan prostaglandin E_2 (PGE_2) telah dikaji menggunakan sel makrofaj RAW264.7 terawat lipopolisakarida (LPS). RT-PCR Kuantitatif telah digunakan untuk menilai ekspresi mRNA bagi COX-1 dan COX-2. Ekspresi protein bagi COX-1 dan COX-2 telah dinilai menggunakan analisis *Western Blot* dan tahap penghasilan PGE_2 telah dinilai menggunakan Asai *Parameter™ PGE_2 (R&D Systems).*

Berdasarkan hasil kajian, mitraginin merencat secara signifikan ekspresi mRNA bagi COX-2 secara dos yang menaik dan diikuti dengan pengurangan penghasilan PGE_2. Sebaliknya, kesan mitraginin terhadap ekspresi mRNA COX-1 adalah tidak signifikan berbanding dengan sel-sel kawalan. Walau bagaimanapun, ekspresi protein COX-1 didapati bergantung kepada kepekatan mitraginin iaitu kepekatan yang lebih tinggi menunjukan perencatan seterusnya bagi ekspresi protein COX-1 terhadap sel-sel terawat dengan LPS. Kesimpulannya, kajian ini menunjukkan mitraginin telah menindas penghasilan PGE_2 dengan merencat ekspresi mRNA COX-2 terhadap sel-sel makrofaj RAW264.7 terawat dengan LPS. Oleh itu, mitraginin didapati mungkin berguna dalam merawat inflamasi.

THE EFFECTS OF MITRAGYNINE FROM *MITRAGYNA SPECIOSA* ON THE mRNA EXPRESSION OF COX-1 AND COX-2 IN LIPOPOLYSACCHARIDE-STIMULATED RAW264.7 MACROPHAGE CELLS

ABSTRACT

Mitragyna speciosa Korth (Rubiaceae) is one of the medicinal plants used traditionally to treat various types of diseases especially in Thailand and Malaysia. Its anti-inflammatory and analgesic properties of its crude form are well documented. In this study, the cellular mechanism involved in the anti-inflammatory effects of mitragynine, the major bioactive constituent, was investigated. The effects of mitragynine on the mRNA and protein expression of COX-1 and COX-2 and the production of prostaglandin E_2 (PGE_2) were investigated in LPS-treated RAW264.7 macrophage cells. Quantitative RT-PCR was used to assess the mRNA expression of COX-1 and COX-2. Protein expression of COX-1 and COX-2 were assessed using Western blot analysis and the level of PGE_2 production was quantified using Parameter™ PGE_2 Assay (R&D Systems).

Based on the results of the studies, mitragynine produced a significant inhibition on the mRNA expression of COX-2 induced by LPS, in a dose dependent manner and this was followed by the reduction of PGE_2 production. On the other hand, the effects of mitragynine on COX-1 mRNA expression were found to be insignificant as compared to the control cells. However, the effect of mitragynine on COX-1 protein expression was dependent on its concentration. Higher concentrations of mitragynine produced a further reduction of COX-1 expression in LPS-treated cells. In conclusion, these findings suggested that mitragynine suppressed PGE_2

production by inhibiting COX-2 expression in LPS-stimulated RAW264.7 macrophage cells. Thus, mitragynine may be useful in the treatment of inflammatory conditions.

CHAPTER 1

INTRODUCTION

1.1 The plant *Mitragyna speciosa* Korth. (Rubiaceae)

1.1.1 Description of the plant

Mitragyna speciosa Korth (Figure 1.1) is a native tropical herb plant belonging to the family Rubiaceae (Coffee family). This species of Mitragyna is found mainly in sub-regions of Asia, particularly in northern parts of Malaysia, central and southern parts of Thailand, and Indonesia (Farah Idayu *et al*, 2011) . In Malaysia, it is popularly known by the local people as "ketum" or "biak-biak" and is mostly found in the states of Perlis, Kedah, Perak, Kelantan, Terengganu and Selangor. It is also well known in Thailand as *"ithang"*, *"thom"*, *"kakuam"* or *"kratom"*. Globally, this plant is known as *"Kratom"*. The genus was named "Korth" after the botanist, William Korthal, who found that the stigma of its flower resembles a bishop's mitre (Shellard, 1974) . This plant can grow to a normal height of 4-9 meters and to a wide of 5 meters. However, certain plants can grow up to 15-30 meters tall. The leaves are normally of dark glossy green colour and can grow over 18 cm long and 10 cm wide with an ovate-acuminate shape and tapered ends (Z. Hassan *et al*, 2013) . The deep yellow flowers grow in globular clusters attached to the leaf axils on long stalks, bearing up to 120 florets each. The seeds are winged (Shellard, 1974; Shellard and Lees, 1965). At present, there are two main varieties of this plant which can be easily distinguished from the leaves. The petiole (vein) could be seen as either red or white-greenish in colour and it was believed that they produced different strength of effects (Murple, 2006).

1.1.2 Chemical constituents of the plant

The chemical constituents of this species especially its alkaloid extract have been well documented years ago. Jansen and Prast (1988) reported that mitragynine

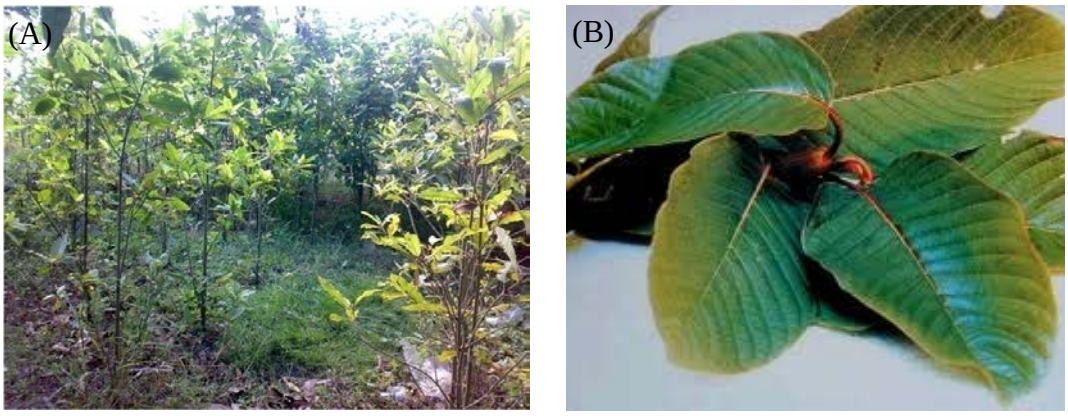

Figure 1.1 *Mitragyna speciosa* plant. (A) Whole plant, (B) Leaves.

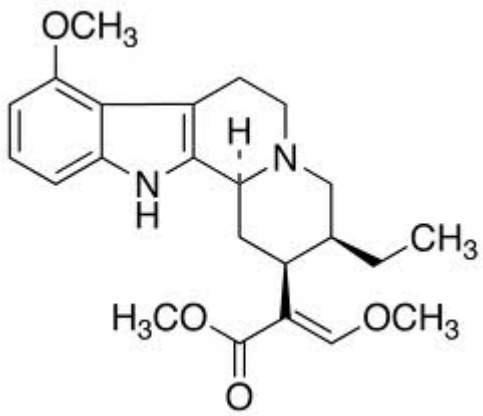

Figure 1.2 Chemical structure of mitragynine

(Figure 1.2) was obtained from the young leaves as the major constituent (66.2% based on the crude base) together with its analogues, speciogynine (6.6%), speciociliatine (0.8%), and paynantheine (8.6%) (Jansen and Prast, 1988). To date, over 20 alkaloids have been successfully isolated and characterized from the leaves (Matsumoto *et al*, 2005; Shellard, 1974; Takayama, 2004; Takayama *et al*, 2000). Mitragynine, the major chemical constituent of interest in this study has a molecular formula of 9-methoxy-corynantheidine ($C_{23}H_{30}N_2O_4$) with molecular weight of 398.50 (Chee *et al*, 2008). The alkaloids profile of *Mitragyna speciosa* is summarized in Table 1.1 (Z. Hassan *et al*, 2013)

1.1.3 Studies on its extracts and bioactive compound mitragynine

Many studies on its extract and bioactive compound mitragynine have been carried out previously. The first pharmacokinetic study of mitragynine was reported by (Janchawee *et al*, 2007) using a HPLC-UV analysis method. It was reported that after oral administration of 40 mg of mitragynine in rats, the peak plasma concentration (C_{max}) was 0.63 µg/ml at time (T_{max}) of 1.83 h. The elimination rate constant (λ_z) was $0.07h^{-1}$ and the clearance was 1.60 L/h. A comprehensive pharmacokinetic profiles of mitragynine in human and rat plasma using a solid-phase extraction and HPLC-UV method was carried out (Parthasarathy *et al*, 2010). After administering 1.5 mg/kg mitragynine intravenously, the C_{max} was 2.3 ± 1.2 µg/mL after (T_{max}) 1.2 ± 1.1 h. The elimination half life ($t_{½}$) was 2.9 ± 2.1 h. The clearance (CL) was 0.29 ± 0.27 L/h/kg. After oral administration of 50 mg/kg mitragynine, the C_{max} was 0.7 ± 0.21 µg/mL after T_{max} 4.5 ± 3.6 h with $t_{½}$ of 6.6 ± 1.3 h. The apparent total CL was 7.0 ± 3.0 L/h/kg. It was found that the bioavailability of mitragynine after oral administration was 3.03 ±1.47%.

Mitragyna speciosa leaves have been used by locals for its opium-like effect and cocaine-like stimulant ability as anti-fatigue, anti-pain and as tonic to increase endurances or performances of work under hot sunlight (Reanmongkol Wantana *et al*, 2007). Mitragynine has also been reported to exhibit antinociceptive as well as analgesic properties. It was demonstrated that mitragynine has antinociceptive actions by suppressing mechanical and thermal noxious stimulations of supraspinal opioid receptors (Matsumoto *et al*, 1996). Later, mu-and kappa-opioid receptor subtypes were found to mediate these actions centrally (Thongpradichote *et al*, 1998). Mitragynine was found to inhibit guinea pig ileum contraction *in vitro* via the opioid receptors (Watanabe *et al*, 1997). It was reported that it also inhibited the vas deferens contraction elicited by nerve stimulation, probably through its blockage of neuronal Ca^{2+} channels (Matsumoto *et al*, 2005).

There were also several toxicity studies on the alkaloid and methanol extracts using animal models. It was reported that 200 mg/kg of total alkaloid extract in rats can be lethal (Azizi *et al*, 2010). Later, an acute oral toxicity study at three different doses (100, 500 and 1000 mg/kg) of standardized methanol extract was found not to have any effect on water and food consumption as well as spontaneous behavior in rats (Harizal *et al*, 2010). However, it was observed that there was a significant increase in alanine transaminase (ALT) and aspartate aminotransferase (AST) at these doses. Nephrotoxicity was observed at 1000 mg/kg as evidenced by elevated creatinine. Histological examinations showed congestion of sinusoids, haemorrhage hepatocytes, fatty change, centrilobular necrosic and increased number of Kuppfer cells in the liver (Harizal *et al*, 2010). It was reported that the methanolic extract of *Mitragyna speciosa* has anti-inflammatory effects in Sprague dawley rats (Raja Aziddin RE *et al*, 2005; Shaik Mossadeq *et al*, 2009). In a recent *in vivo* study,

mitragynine has shown to exert an antidepressant effect in animal behavioral model depression (FST and TST) and the effect appears to be mediated by an interaction with neuroendocrine HPA axis systems (Farah Idayu *et al*, 2011) .

Table 1.1 The estimated percentage of compounds in the alkaloid extracts of *Mitragyna speciosa* Korth (Z. Hassan *et al*, 2013)

Alkaloids	Percentage	Effects	References
Mitragynine	66%	Analgesic, antitussive, antidiarrheal, adrenergic, antimalarial	(Field, 1921; Hooper, 1907; Lee *et al*, 1967; Ponglux *et al*, 1994)
Paynantheine	9%	Smooth muscle relaxer	(Ponglux *et al*, 1994)
Speciogynine	7%	Smooth muscle relaxer	(Lee *et al*, 1967; Ponglux *et al*, 1994; Shellard, 1974; Shellard *et al*, 1978b)
7-Hydroxymitragynine	2%	Analgesic, antitussive, antidiarrheal	(Ponglux *et al*, 1994)
Speciociliatine	1%	Weak opioid agonist	(Lee *et al*, 1967; Ponglux *et al*, 1994)
Mitraphylline	<1%	Vasodilator, antihypertensive, muscle relaxer, diuretic, antiamnesic, immunostimulant, anti-leukemic	(Ponglux *et al*, 1994; Seaton *et al*, 1958; Shellard, 1974; Shellard *et al*, 1978b)
Isomitraphylline	<1%	Immunostimulant, anti-leukemic	(Ponglux *et al*, 1994; Seaton *et al*, 1960; Shellard and Philipson, 1966)
Speciophylline	<1%	Anti-leukemic	(Beckett *et al*, 1966; Shellard and Philipson, 1966)
Rhynchophylline	<1%	Vasodilator, antihypertensive, calcium channel blocker, antiaggregant, anti-inflammatory, antipyretic, anti-arrhythmic, antihelminthic	(Seaton *et al*, 1960; Shellard, 1974; Shellard *et al*, 1978b)

Table 1.1 *Continued*

Alkaloids	Percentage	Effects	References
Isorhynchophylline	<1%	Immunostimulant	(Seaton *et al*, 1960; Seaton *et al*, 1958; Shellard, 1974; Shellard *et al*, 1978b)
Ajmalicine	<1%	Cerebrocirculant, antiaggregant, anti-adrenergic, sedative, anticonvulsant, smooth muscle relaxer	(Beckett *et al*, 1966)
Corynantheidine	<1%	Opioid agonist	(Takayama *et al*, 2002)
Corynoxine A	<1%	Calcium channel blocker, anti-locomotor	(Shellard *et al*, 1978a)
Corynoxine B	<1%	Anti-locomotor	(Shellard *et al*, 1978a)
Mitrafoline	<1%		(Hemmingway *et al*, 1975; Shellard *et al*, 1978a)
Isomitrafoline	<1%		(Hemmingway *et al*, 1975; Shellard *et al*, 1978a)
Oxindale A	<1%		(Shellard *et al*, 1978a)
Oxindole B	<1%		(Shellard *et al*, 1978a)
Speciofoline	<1%	Analgesic, antitussive	(Hemmingway *et al*, 1975)
Isospeciofoline	<1%		(Hemmingway *et al*, 1975; Shellard *et al*, 1978a)
Ciliaphylline	<1%	Analgesic, antitussive	(Trager *et al*, 1968)
Mitraciliatine	<1%		(Lee *et al*, 1967)
Mitragynaline	<1%		(Houghton *et al*, 1991)
Mitragynalinic acid	<1%		(Houghton *et al*, 1991)
Corynantheidalinic acid	<1%		(Houghton *et al*, 1991)

1.2 Inflammation and pain

1.2.1 Inflammation

The word inflammation is derived from the Latin “inflammare” (to burn). It is one of the most important biological responses involved in the defense of an organism against local injury and harmful stimuli such as pathogens or irritants. It often progresses to painful or chronically harmful inflammatory diseases requiring appropriate medicinal treatment (Laupattarakasem *et al*, 2003; Vane *et al*, 1994). Typical inflammatory diseases such as rheumatoid, asthma, colitis and hepatitis are among the leading causes of death and disability in the world (Cirino *et al*, 2003; Emery, 2006; Jiang and Ames, 2003). The development of several inflammatory diseases including cancer, cardiovascular and neurodegenerative disorders are due to chronic inflammation (Jiang and Ames, 2003; Willerson and Ridker, 2004).

Inflammatory response is a series of well coordinated dynamic mechanism consisting of specific vascular, humoral and cellular events that is characterized by movement of fluids, plasma and inflammatory leukocytes (neutrophils, eosinophils, basophils and macrophages) to the site of inflammation (Gokhale *et al*, 2002; Hou *et al*, 2004). A variety of chemical mediators or signaling molecules such as histamines, serotonin, leukotrienes, prostaglandins and oxygen derived free radicals are produced by inflammatory and phagocytic cells predominantly in the sequences which participate in onset of inflammation (Safayhi and Sailer, 1997; Vijayalakshmi *et al*, 1997). Inflammatory response occurs in two phases which are known as acute and chronic and each is apparently mediated by a different mechanism.

1.2.1.1 Acute inflammation

Acute inflammation is the immediate defence to tissue-specific damage. The cardinal signs of acute inflammation are those described by Celsus in the 1st century AD as rubor (redness), calor (heat), tumor (swelling) and dolar (pain) (Nathan, 2002). These symptoms arise due to dilation of blood vessels, causing increased blood flow and migration of white blood cells to the affected area, and they terminate once the cells are properly healed. The events involved in acute inflammation can be divided into vascular and cellular.

1.2.1.1.1 Vascular events

Vascular events occur in micro vasculature and become apparent in 15-30 minutes after tissue injury, infection and in the presence of other inflammatory stimuli. It is mainly mediated by chemicals such as serotonin and histamine released from mast cells. It is actually a transient phase and is characterized by local vasodilations of venules and capillaries resulting in an increased blood flow to the inflamed tissue, thereby giving rise to localized redness and heat followed by an increase in vascular permeability leading to transudation of fluids, plasma and vascular protein into the inflammatory sites producing interstitial oedema (Gallin *et al*, 1992; Nathan, 2002).

1.2.1.1.2 Cellular events

The infiltration of leukocytes from circulating blood is crucial in inflammatory reaction (Muller, 2002; Negrotto *et al*, 2006). A variety of chemotactic agents such as bacterial products possessing amino terminal N-formyl methionyl groups, C5a complement fragment and chemokines along with mediators of mast cells like histamine and leukotriene B_4 (LTB4) and platelets activating factor (PAF)

elicit profound leukocytes infiltration within 30-60 minutes (Asako *et al*, 1992; Raud *et al*, 1989). The first inflammatory cells or leukocytes that are recruited at the site of acute inflammation are known as neutrophils (Hou *et al*, 2004; Miyazaki *et al*, 2000). Cell infiltration occurs through a process in which leukocytes interact with endothelium in post capillary venules. This process involves its sequential capture, rolling along and firm adhesion to the micro vascular endothelium, followed by transmigration through the vessel wall and further migration in extravascular tissue (Muller, 2002). All these steps in the recruitment cascade are controlled by cell adhesion molecules (CAM) such as selectin, integrins (CD11 and CD18) and intracellular adhesion molecules (ICAM-1 and -2). There are three members of the selectin family of CAMs which are expressed on both leukocytes (L-selectin) and endothelial cells (P-selectin and E-selectin). These molecules mediate low affinity adhesion of leukocytes and endothelial cells during rolling process (Vestweber and Blanks, 1999). High-affinity adhesion molecules of leukocytes on endothelium are mediated by interaction between integrins (CD11/CD18) and adhesion molecules (CAM-1 and CAM-2) expressed on leukocytes and endothelium cells, respectively (Ulbrich *et al*, 2003). Following a period of stationary adhesion, a leukocyte may leave the post capillary venules by extending pseudopodia between endothelial cells and reach into the subendothelial space. This complex process is often known as leukocytes extravasations and transendothelial migration.

1.2.1.2 Chronic inflammation

Chronic inflammation is characterized by infiltration of mononuclear cells such as macrophages and lymphocytes, proliferation of fibroblast, collagen fibers and formation of connective tissue which ultimately lead to 0.5 – 2.0 mm large

granuloma (tumour like swelling). In this situation tissue degeneration is mainly mediated by reactive oxygen and nitrogen species and proteases produced from infiltrated inflammatory cells (Suleyman *et al*, 2004). These reactive oxygen species are mutagenic and during the process of repeated tissue damage and regeneration, they interact with DNA in proliferating epithelium resulting in permanent genomic alterations such as point mutations, deletions or rearrangements (Maeda and Akaike, 1998). Indeed, p53 mutations are seen in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel diseases at frequencies similar to those in tumours (Yamanishi *et al*, 2002). Thus, chronic inflammation ultimately, progress to carcinoma (Coussens and Werb, 2002).

1.2.2 Pain

Pain normally serves as an alarm system activated in response to impending damage to the organism. It is an unpleasant sensory and emotional experience associated with actual or potential tissue damage as per the International Association for the Study of Pain (IASP) (Merskey and Bogduk, 1994). It is a multidimensional experience, which contains essentially a sensory, cognitive and emotional component (Woolf, 2004). Pain can be classified into different categories according to various criteria, such as those based on the cause of pain, its duration, location, underlying diseases etc. The most widely used criteria are aetiological (i.e. based on the cause of pain). In this regard, pain can be classified into three categories, nociceptive, inflammatory and neuropathic. Nociceptive pain is generated by activation of noniceptors that are specialized to be activated by noxious stimuli which have the potential of causing tissue damage. Nociception or nociceptive pain is essential in survival for organisms to avoid potential or actual tissue damage (Scholz and Woolf,

2002). Nociceptive pain is mostly recognized as acute pain since that pain stops when the stimulus has been removed.

Inflammatory pain is associated with processes that can be caused by tissue damage, infections, tumor growth and various forms of chronic inflammatory diseases, such as autoimmune disease. During inflammation, multiple mediators are released locally from the damaged and recruited inflammatory cells. This results in the release of cytokines, growth factors, neuropeptides, kinins, purines, amines, prostanoids and ions, including protons (Boddeke, 2001; Mantyh *et al*, 2002). These mediators can activate and sensitize nociceptors, thus evoke pain (Scholz and Woolf, 2002). The symptoms of inflammation include cell migration, oedema, erythema, pain and hyperalgesia (Marchand *et al*, 2005). In most cases, inflammatory pain responds to non-steroidal anti-inflammatory drugs (NSAIDs) or opiates such as morphine (Fitzcharles *et al*, 2010). Inflammatory pain under many conditions such as rheumatoid arthritis (RA) is chronic. Chronic inflammatory pain can be characterized by hyperalgesia (greater pain after normally painful stimuli) and allodynia (normally non-noxious stimuli that are perceived as painful).

Neuropathic pain arises from a primary lesion or dysfunction in the peripheral or central nervous system (CNS) such as painful polyneuropathy, postherpetic neuralgia, trigeminal neuralgia, spinal cord injury pain and post-stroke pain. (Merskey and Bogduk, 1994). Clinically, neuropathic pain is characterized by spontaneous on-going or shooting pain and evoked pain such as hyperalgesia and allodynia (Baron *et al*, 2010). Neuropathic pain is mostly chronic, difficult to treat and associated with plasticity in the central and peripheral nervous system. The mechanisms of neuropathic pain are not well understood and treatments are largely unsatisfactory. Neuropathic pain may respond to some antiepileptics, tricyclic

antidepressant, and antiarrhythmics. Local anesthetics used to block the nerve may also be effective in some cases.

1.3 Molecular mechanisms of inflammation and pain

1.3.1 Cyclooxygenase (COX)

Cyclooxygenase (COX), also known as prostaglandin-endoperoxide synthase, is a heme-containing enzyme involved in the metabolism of arachidonic acid (AA) and the synthesis of prostanoids including potent pro-inflammatory prostaglandins (PGE_2, PGF2α) (Hood *et al*, 2003; Mitchell *et al*, 1993). In mammalian cells, COX exist in at least two isoforms COX-1 and COX-2 (Fu *et al*, 1990; Langenbach *et al*, 1995; Xie *et al*, 1991). It is generally considered that COX-1 is constitutively expressed in almost all cell types, including platelets and those present in the stomach, kidney, vascular endothelium, forebrain and uterine epithelium and is regulated as a house keeping enzyme for various physiological functions. COX-2 is inducible and expressed during tissue damage or inflammation in response to pro-inflammatory cytokines such as interleukin-1-beta (IL-1β), interferon gamma and tumor necrosis factor-alpha (TNF-α) (Akarasereenont *et al*, 1994; Arias-Negrete *et al*, 1995; Hood *et al*, 2003; Warner *et al*, 1999). Therefore, COX-2 has been implicated in pathogenesis, such as inflammation, pain, fever and cancer (Murakami and Kudo, 2004).

The human cyclooxygenase genes have been cloned and assigned to different chromosomes, the COX-1 gene is present on chromosome 9 and the COX-2 on chromosome 1 (Kosaka *et al*, 1994; Tazawa *et al*, 1994). The COX-1 gene contains 11 exons in 22 kb and COX-2 gene has 10 exons in 8.3 kb (Kraemer *et al*, 1992). Both isoforms of cyclooxygenase are structurally distinct proteins, the amino acid

sequence of their complementary DNA showing approximately 60% homology (Kurumbail *et al*, 1996). COX-1 contains 576 amino acids and COX-2 contains 587 amino acids with a molecular mass of ~71 kDa. Although both the isozymes have similar active site for their natural substrate arachidonic acid (AA), slight differences between the two isozymes were evident in 3-D structural analysis. In COX-1 the 523 position is occupied by an isoleucine, while in COX-2 the same position is occupied by a valine residue which is different by a single methyl group. The smaller valine residue in COX-2 produced a large gap in the enzyme channel, giving access to site pocket which is thought to be the binding site of many selective COX-2 inhibitory agents (Cannon *et al*, 1998; Lanzo *et al*, 1998; Marnett and Kalgutkar, 1999; Wong *et al*, 1997).

1.3.2 Prostaglandins biosynthesis

The COX isozymes are integral membrane proteins. Arachidonic acid that is released from the membrane adjacent to the opening of the enzyme channel, is attracted to its hydrophobic region and later transformed into prostaglandins and other related metabolites. COX- enzymes possess two distinct catalytic activities: a *cyclooxygenase activity* which catalyzes the formation of a C-5 ring molecule called PGG_2 by reacting with two molecules of oxygen and a peroxidase activity in which the peroxide group at C-15 is reduced to an alcohol with the formation of PGH_2 (Funk, 2001; Pulichino *et al*, 2006). PGH_2 is the precursor for the different biologically active prostaglandins and thromboxanes (Vane *et al*, 1998) (Figure 1.3). Several isomerases such as PGD synthase, PGF synthase and PGE synthase catalyze the transformation of PGH_2 into different prostaglandin PGD_2, $PGF_{2\alpha}$, PGE_2 respectively. Prostacyclin synthase catalyzes the transformation of PGH_2 into

prostacyclin (PGI_2) and thromboxane synthase carries out the transformation of PGH_2 into thromboxanes A_2.

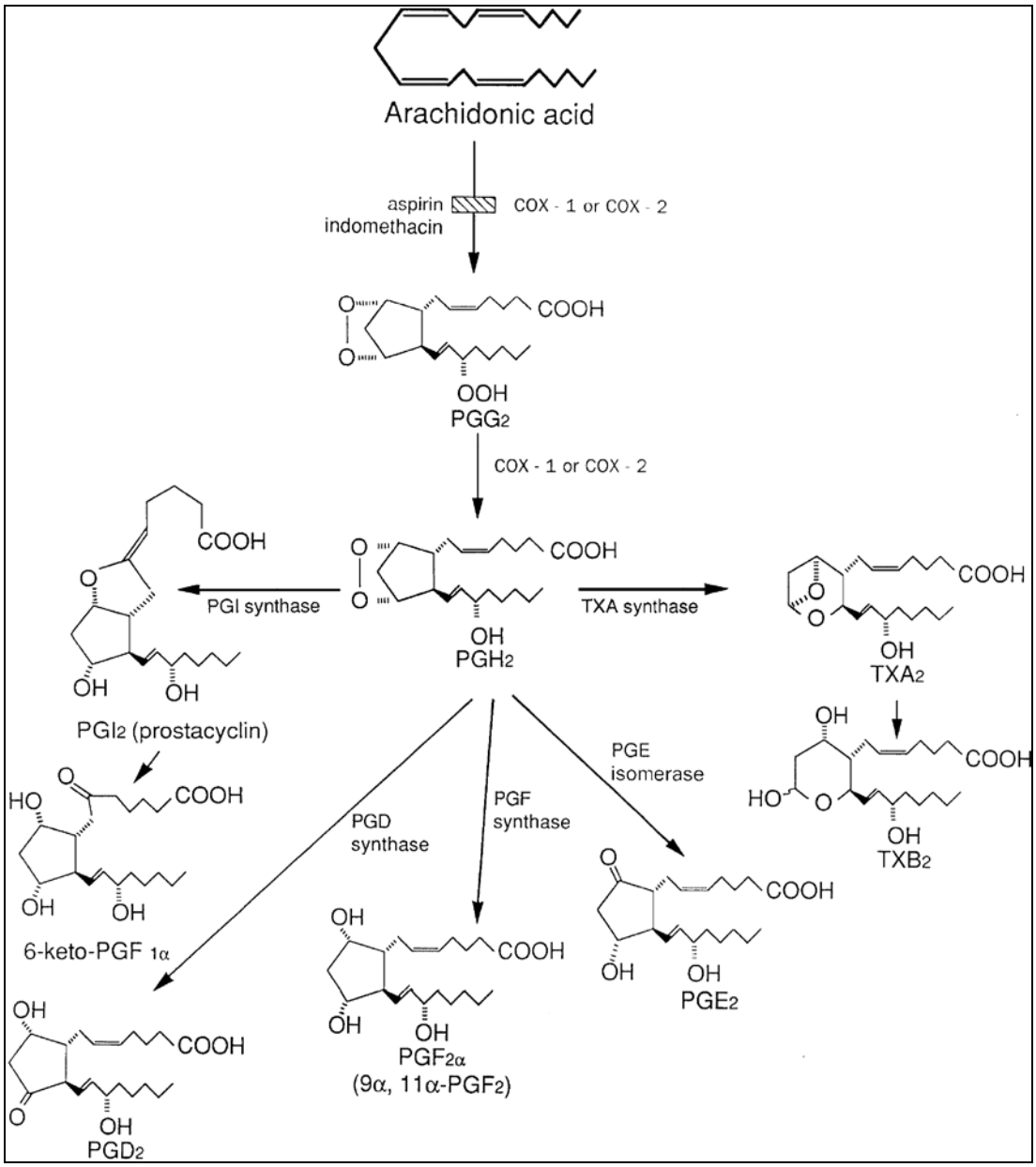

Figure 1.3 The arachidonic acid cascade and prostaglandins biosynthesis (Vane *et al*, 1998)

1.3.3 Physiological and pathophysiological role of prostaglandins

Prostanoids formed by COX-1 are important in many physiological functions including the regulation of platelet aggregation. Thromboxane TXA_2 induces platelet aggregation while PGI_2 exhibits antiaggregatory properties. In the gastrointestinal tract, PGI_2 and PGE_2 reduce gastric acid secretion, exert a direct vasodilator action on the vessels of the gastric mucosa and stimulate the production of viscous mucus which forms a protective barrier (Vane and Botting, 1998). In the kidney, vasodilator prostaglandins (PGI_2, PGE_2 and PGD_2) play a key role in regulating renal blood flow, diminishing vascular resistance, dilating renal vascular beds and enhancing organ perfusion (Whelton, 1999). COX-1 is found in neurons throughout the brain, but is most abundant in the forebrain where PGs may be involved in complex integrative functions (Breder *et al*, 1995; Yamagata *et al*, 1993). It is also expressed in the uterine epithelium in early pregnancy and may be important for implantation of the ovum and for angiogenesis necessary to establish the placenta (Chakraborty *et al*, 1996).

On the other hand, prostaglandins (PGE_2 and PGI_2) are substantially involved in maintaining the inflammatory process by increasing vascular permeability and amplifying the effect of other inflammatory mediators such as kinin, serotonin and histamine. Thus, contributing to the redness, increased blood flow and plasma exudation in the area of acute inflammation which leads to oedema. These PGs produce hyperalgesia by sensitizing afferent C fibers. Moreover, PGE_2 acts on neurons in the thermoregulatory network of the hypothalamus, causing increase in body temperature. Elevated levels of multiple PGs including PGE_2 and PGI_2 have been reported in synovial fluids from patients with rheumatoid arthritis and osteoarthritis (Egg, 1984; Pulichino *et al*, 2006). Prostaglandins also play an

important role in the pathogenesis of several types of cancers such as breast, liver and lung with over expression of COX-2 and over production of prostaglandin (Achiwa *et al*, 1999; DuBois, 2001; Hwang *et al*, 1998).

Pain and inflammation are mediated by PGE_2 and PGI_2 through their actions on various receptors. PGE_2 G-protein coupled receptor subtypes (EP1, EP2, EP3 and EP4) and PGI_2 receptor (IP) have been identified (Lin *et al*, 2006; McCoy *et al*, 2002; Pulichino *et al*, 2006). At the site of inflammation, PGE_2 sensitizes peripheral pain through activation of EP receptors present on the peripheral terminals of sensory neurons (Lin *et al*, 2006). Similarly, in another study PGI_2 receptor deficient mice display an impaired acute inflammatory response in carrageenan-induced paw oedema and acetic acid-induced writhing (Murata *et al*, 1997), indicating the participation of PGI_2 receptor in inflammation.

1.4 Therapeutic management for inflammation and pain

1.4.1 Nonsteroidal Anti-inflammatory Drugs (NSAIDs)

Inflammation and pain are currently regularly treated by non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are the most commonly used over-the-counter drugs (A Mahajan and Rashmi Sharma, 2005). Aspirin (acetylsalicylic acid) is one of the NSAIDs available in the market since 1899. It was introduced by the chemist Felix Hoffman (Wallace, 1997). The 1960s was a key decade for the discovery of various classes of NSAIDs essential for the treatment of pain and inflammation. A significant advancement in the evaluation of NSAIDs was made by two important discoveries. Firstly, the substantial progress made in 1970s in elucidating the mechanism of action of NSAIDs related to inhibition of prostaglandins by cyclooxygenase pathway. Secondly, the identification of two

isoforms of cyclooxygenase (COX) in the 1990s (Lin *et al*, 2006; Vane and Botting, 1998; Warner *et al*, 1999). NSAIDs can act as nonselective COX inhibitors or selective COX-2 inhibitors based on its selectivity towards cyclooxygenases.

1.4.2 Non-selective cyclooxygenase inhibitors

Non-selective NSAIDs belong to a heterogenous group of chemical substances that inhibit both constitutively expressed COX-1 and the inducible COX-2 almost with the equal potency. Most of the NSAIDs are carboxylic acid containing drugs such as salicylate derivatives (e.g. aspirin), carboxylic and heterocyclic acid derivatives (e.g. indomethacin), fenamic acid derivatives (e.g. mefenamic acid), propionic acid derivatives (e.g. ibuprofen, ketoprofen, flurbiprofen and naproxen) and phenyl acetic acid derivatives (e.g. diclofenac). NSAIDs having enolic acid containing drugs include oxicam derivatives (e.g. piroxicam, tenoxicam and meloxicum) and pyrazoles (e.g. phenylbutazone). These organic acid containing drugs act at the site of the enzyme and interact with the guanidinium group of Arg-120, thereby preventing the access of AA to the enzyme and so stop the cyclooxygenase pathway (Derle *et al*, 2006; Mancini *et al*, 1995).

The classical NSAIDs (aspirin like drugs) are among the most widely prescribed drugs worldwide as analgesic, antipyretic and anti-inflammatory agents and have become an important drug in the control of inflammation and pain associated with musculoskeletal pathologies such as rheumatoid arthritis, osteoarthritis, gout tendonitis, muscle strain, postoperative and post traumatic inflammation, thrombophlebitis and vasculitis (Pulichino *et al*, 2006; Smith *et al*, 1998; Steinmeyer, 2000; Warner *et al*, 1999).

1.4.3 COX-2 inhibitors

Considering the side effects of classical NSAIDs, a selective blockade of the COX-2 isoform would lead to the inhibition of pain and inflammation without impending the COX-1 dependent affect in the GI tissue and kidney (Hawkey, 1999; van Ryn and Pairet, 1999). Thus in 1999, rofecoxib (Vioxx) and celecoxib (Celebrex) were developed as a leading anti-arthritic drug with 800 and 375 fold more selectivity, respectively towards COX-2, than COX-1. Clinical studies revealed that both drugs possess similar efficacy to diclofenac and naproxen but there was a lower incidence of gastrointestinal adverse effect in prolonged use (Alvaro-Gracia, 2004; Bertolini *et al*, 2002; Cannon *et al*, 1998; van Ryn and Pairet, 1999). However, various clinical studies have raised concern about the cardiovascular safety in the use of selective COX-2 inhibitors, as long term use of these inhibitors increase the risk of myocardial infarction and stroke in patients with rheumatoid arthritis (Bombardier *et al*, 2000; Bresalier *et al*, 2005). This cardiac risk is mainly attributed to the inhibition of prostacyclin (PGI_2) which would cause a severe physiological imbalance between prothrombotic thromboxane A_2 (levels are raised) and vasodilatory prostacyclin (decline) levels in the endothelium, favoring platlet aggregation and vasoconstriction (Lin *et al*, 2006; Linton and Fazio, 2004). As a result of which in September 2004 the use of Vioxx was banned in the treatment of rheumatoid arthritis.

1.4.4 Opioids

Opioids is one of the treatments given to patients in managing pain. Opioids have an important role in acute pain management of moderate to severe pain, but the dangers of chronic use have long been of concern. The following information about the mechanism of action of opioids is excerpted from Nicholson 2003. Opioids

mediate their actions by binding and activating endogenous opioid receptors that comprise part of a pain-modulating pathway that descends from the midbrain to the spinal cord dorsal horn. Opioids receptors and endogenous opioid peptides have also been identified in the peripheral nervous system. Opioid receptors consist of three subtypes: mu (μ), delta (δ) and kappa (κ). The pharmacological effects of the opioid analgesics are derived from their complex interactions with these three opioid receptors (Inturrisi, 2002). Most opioid drugs, for which morphine is the prototype, are relatively selective for μ-receptors. These drugs are full agonists and through their stimulation of μ-receptors produce analgesia, affect mood and rewarding behavior, and alter respiratory, cardiovascular, gastrointestinal, and neuroendocrine functions.

In recent years, this notion has been challenged or de-emphasized, and many clinicians who treat chronic pain have assumed that maintenance opioids retain analgesic efficacy despite a lack of good evidence for this assumption (Streltzer and Johansen, 2006). Chronic stimulation of the μ-opioid receptor results in a cascade of cellular responses with multiple overlapping mechanisms, which can result in enhanced pain sensitivity, known as hyperalgesia (Chang *et al*, 2007). Some of the cellular responses to chronic opioid intake that are thought to contribute to hyperalgesia include an increase in neuropeptides such as dynorphin (Vanderah *et al*, 2001), cholecystokinin (Xie *et al*, 2005), and substance P (King *et al*, 2005) all of which have been demonstrated to enhance pain sensitivity and the activation of glial cells, producing inflammatory cytokines and resulting in amplified pain (Watkins *et al*, 2007).

1.5 Objectives of the study

At present, the studies on anti-inflammatory and analgesic properties of extracts of *Mitragyna speciosa* are well documented; however, the cellular mechanisms involved in the anti-inflammatory effects of mitragynine, the major bioactive constituent has yet to be elucidated. Therefore, this study is important for our understanding on the involved cellular mechanisms specifically on the modulation of COX-1 and COX-2 genes and protein expression as cyclooxygenase (COX) enzymes play an important role in the induction of pain and inflammation as well as the analgesic actions of NSAIDs. COX-1 is constitutively expressed in almost all cell types, including platelets and those present in the stomach, kidney, vascular endothelium, forebrain and uterine epithelium and is regulated as a house keeping enzyme for various physiological functions. COX-2 is inducible and expressed during tissue damage or inflammation in response to pro-inflammatory cytokines. The objectives of the present study are to determine:

1. The cytotoxicity effects of mitragynine in RAW264.7 macrophage cells.
2. The effects of mitragynine on mRNA and protein expression of COX-1 and COX-2 in LPS-stimulated RAW264.7 macrophage cells.
3. The effects of mitragynine on Prostaglandin E_2 (PGE_2) production in LPS-stimulated RAW264.7 macrophage cells.

CHAPTER 2

MATERIALS AND METHODS