ANTI-INFLAMMATORY AND ANALGESIC ACTIVITIES OF GARCINIA ATROVIRIDIS FRUIT EXTRACTS

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ANTI-INFLAMMATORY AND ANALGESIC ACTIVITIES OF GARCINIA ATROVIRIDIS FRUIT EXTRACTS

By

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This thesis is dedicated to

my beloved & supportive

family

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LIST OF ABBREVIATIONS

^{o}C	degree Celsius
%	Percent
$\%B/B_o$	% Bound/Maximum Bound
5-HT	Serotonin
ANOVA	Analysis of variance
ARASC	Animal Research and Service Centre
A.U	Arbitary Unit
B_o	Maximum binding
β2	Beta-2
BK	Bradykinin
blk	Blank
cAMP	Cyclic adenosine monophosphate
cc	cubic centimeters
CE	Chloroform extract
CF	Chloroform fraction
CNS	Central nervous system
CO_2	Carbon dioxide
COX-1	Cyclooxygenase-1
COX-2	Cycloxygenase-2
cPGES	Cytosolic prostaglandin E_2 synthase
δ	delta
DC	Dendritic cell
DHC	Dihydrocodeine
DHC-6-G	Dihydrocodeine-6-glucuronide
DHM	Dihydromorphine
DMSO	Dimethyl sulfoxide
dtn	decitonne
e.g.	Example
EAF	Ethyl acetate fraction
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunoassay
EP1, EP2, EP3, EP4	Prostaglandin E_2 receptors
FBS	Fetal bovine serum
$FeCl_3$	Iron chloride
g	gram
GA	Garcinia atroviridis
GPCRs	Super family G protein-coupled receptors
H_1R, H_2R, H_3R, H_4R	Histamine receptors
H_2SO_4	Sulphuric acid
H	Histamine
HCA	Hydroxycitric acid
HCAL	Hydroxycitric acid lactone
HCL	Hydrochloric acid
HMW	High molecular weight
HRP	Horse radish peroxide

IC_{50}	Half maximal inhibitory concentration
i.p	intraperitoneal
IND	Indomethacin
iNOS	inducible Nitric oxide synthase
IL	Interleukin
IP	Prostanoid receptor
κ	kappa
kg	kilogram
KO	Knockout
L	Liter
LOX	Lipoxygenase
LPS	Lipopolysaccharide
Lts	Leukotrienes
LMW	Low molecular weight
ME	Methanol extract
mPGES	membrane prostaglandin E_2 synthase
µ	mu
µl	microliter
MAPK	Mitogen-activate protein kinase
mg	milligram
MGC	Multinucleated gigantic cell
MN	Mononuclear
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NaCl	Sodium chloride
nBF	n-butanol fraction
NDHC	Nordihydrocodeine
NF-κβ	Nuclear factor-kappa beta
nm	nanometer
NSAIDs	Non-steroidal antiinflammatory drugs
NSB	Non-specific binding
OD	Optical density
p.o	per os (oral)
p/s	penicillin/streptomycin
PAF	Platelet activating factor
PE	Petroleum ether extract
PLA_2	Phospholipase A_2
PMA	Phorbol 12-myristate 13-acetate
PMNL	Polymorphonuclear leukocytes
PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGF2α	Prostaglandin F2α
PGG/H	Prostaglandin endoperoxide
PGI	Prostacylin
PKC	Protein kinase C
rpm	revolution per minutes
RPMI	Roswell Park Memorial Institute
SD	Sprague Dawley

S.E.M	Standard error mean
T80	Tween 80
T_H	Helper T
TA	Total activity
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF-α	Tumor necrosis factor-alpha
TPA	Tetradecanoyl phorbol acetate
TXB	Thromboxane
U/ml	Units/milliliter
URTI	Upper respiratory tract infection
VSCCs	Voltage sensitive calcium channels
WE	Water extract
WF	Water fraction
w/v	weight/volume

AKTIVITI ANTI-INFLAMASI DAN ANALGESIA EKSTRAK BUAH GARCINIA ATROVIRIDIS

ABSTRAK

Garcinia atroviridis (GA) merupakan herba perubatan boleh dimakan yang digunakan secara tradisional dalam merawat sakit telinga, perit tekak, batuk, kelemumur, bengkak abdomen dan rawatan selepas bersalin. Dalam kajian ini, aktiviti anti-inflamasi dan analgesia berpandukan pengekstrakan dan fraksinasi buah GA telah dilakukan dalam percubaan untuk mengenalpasti agen anti-inflamasi dan analgesia yang lebih poten dan mempunyai kurang kesan sampingan. Buah GA dihiris, dikeringkan, dikisar dan diekstrak secara bersiri dengan petroleum eter, kloroform, metanol dan air melalui kaedah maserasi. Ekstrak dikeringkan di bawah tekanan terturun dan kemudian dibekukering. Hasil ekstrak buah GA adalah 1.45 %, 0.89%, 21.24% dan 7.99% bagi ekstrak petroleum eter (PE), kloroform (CE), metanol (ME) dan air (WE), masing-masing. Aktiviti anti-inflamasi bagi ekstrak-ekstrak dikaji menggunakan model tapak kaki belakang tikus aruhan karagenan. Didapati bahawa pemberian oral ME pada dos 500 dan 1000 mg/kg merencat edema kaki belakang tikus secara signifikan. ME kemudiannya difraksikan kepada fraksi klorofom (CF), fraksi etil asetat (EAF), fraksi n-butanol (nBF) dan fraksi air (WF). Antara empat fraksi, didapati WF memberikan kesan yang paling ketara dalam merencatkan pertumbuhan edema. Aktiviti anti-inflamasi bagi WF terus dikaji menggunakan kapas pelet granuloma dalam tikus, model inflamasi sub-kronik. WF pada (125, 250 and 500 mg/kg) merencat pembentukan granuloma ($p<0.001$). Analisis cytokine dalam darah menunjukkan paras TNF-α dan IL-1 dalam tikus yang

dirawat secara oral dengan WF adalah lebih rendah secara signifikan berbanding kumpulan kawalan. Aktiviti analgesia bagi pemberian ekstrak secara oral dikaji menggunakan ujian plat panas dan "writhings" teraruh asid asetik terhadap tikus. Dalam ujian plat panas, tiada ekstrak GA yang meningkatkan masa tindakbalas tikus terhadap haba secara signifikan. Namun begitu, pemberian PE, CE dan ME (500 mg/kg) merencatkan tindakbalas "writhings" secara signifikan ($p<0.001$). Ini mencadangkan bahawa tiga ekstrak memiliki aktiviti analgesia sama dengan non-steroidal anti-inflammatory drugs (NSAIDs). Memandangkan ME menunjukkan aktiviti analgesia yang tertinggi, ia difraksikan kepada fraksi kloroform (CF), fraksi etil asetat (EAF), fraksi n-butanol (nBF) dan fraksi air (WF). WF telah dijumpai sebagai yang paling ketara dalam merencat "writhings". Penglibatan cyclooygenase (COX-1 dan COX-2) dan cytokine (TNF-α dan IL-1) dalam mekanisma anti-inflamasi bagi WF telah dikaji. WF (200 µg/ml) telah dijumpai merencat kedua-dua COX-1 dan COX-2. WF pada kepekatan melebihi 100 µg/ml telah dijumpai sebagai sitotosik kepada sel-sel U937. Oleh itu, WF pada kepekatan yang lebih rendah (50 µg/ml dan 25 µg/ml) telah digunakan bagi mengkaji kesan WF terhadap pelepasan TNF-α dan IL-1 oleh sel-sel U937. WF telah dijumpai merencat pelepasan TNF-α dan IL-1. Ini mencadangkan bahawa mekanisma tindakan anti-inflamasi bagi WF antaranya adalah dengan merencatkan COX-1, COX-2, TNF-α dan IL-1. Saringan fitokimia menunjukkan kehadiran flavanoid, alkaloid dan saponin dalam WF. Analisa High Performance Liquid Chromatography (HPLC) menunjukkan kehadiran hydroxycitric acid dan kumpulan laktonnya dalam ME dan WF bagi ekstrak buah G. atroviridis.

ANTI-INFLAMMATORY AND ANALGESIC ACTIVITIES OF GARCINIA ATROVIRIDIS FRUIT EXTRACTS

ABSTRACT

Garcinia atroviridis (GA) is an edible medicinal herb traditionally used for the treatment of earache, throat irritation, cough, dandruff, swollen abdomen and postpartum medication. In the present study, the anti-inflammatory and analgesics activity-guided extraction and fractionation of GA fruit were carried out in an attempt to identify anti-inflammatory and analgesic agents which are more potent and with less side effects. The GA fruits were sliced, dried, pulverized and serially extracted with petroleum ether, chloroform, methanol and water using maceration method. The extracts were dried under reduced pressure and freeze-dried. The extraction yield were 1.45%, 0.89%, 21.24% and 7.99% petroleum ether (PE), chloroform (CE), methanol (ME) and water (WE) extracts, respectively. The anti-inflammatory activity of the extracts was studied using carrageenan-induced hind paw oedema in rat. Only administered ME at doses of 500 and 1000 mg/kg were found significantly inhibited the hind paw oedema. ME was then fractionated into chloroform (CF), ethyl acetate (EAF), n-butanol (nBF) and water (WF) fractions. Among these four fractions, WF was found to be the most potent in inhibiting the oedema formation. The anti-inflammatory activity of WF was further studied in cotton pellet-induced granuloma in rats, a sub-chronic model of inflammation. WF (125, 250 and 500 mg/kg) significantly inhibited the granuloma formation ($p<0.001$). Blood cytokine analysis showed that the level of TNF-α and IL-1 in the WF orally treated rats were significantly lower than the control group. The analgesic activity of

the orally administered extracts was studied using hot plate and acetic acid-induced writhings tests in mice. In the hot plate test, none of the GA extracts significantly increased the mice response time to heat. However, administration of PE, CE and ME (500 mg/kg) significantly inhibited writhings response ($p<0.001$). It suggests that the three extracts possess analgesic activity similar to non-steroidal anti-inflammatory drugs (NSAIDs). Since the ME showed the highest analgesic activity, it was fractionated to chloroform (CF), ethyl acetate (EAF), n-butanol (nBF) and water (WF) fractions. WF was found to be the most potent in inhibiting the writhings. The involvement of cyclooxygenases (COX-1 and COX-2) and cytokines (TNF-α and IL-1) in the anti-inflammatory mechanism of WF were studied. WF (200 µg/ml) was found to inhibit both COX-1 and COX-2. WF at concentrations above 100 µg/ml was found to be cytotoxic to U937 cells. Therefore, the lower concentrations of WF (50 µg/ml and 25 µg/ml) were used to study the effect of WF on TNF-α and IL-1 released by U937 cells. WF was found to inhibit the released of TNF-α and IL-1. It suggests that the anti-inflammatory mechanism of actions of WF among others are by inhibiting COX-1, COX-2, TNF-α and IL-1. Phytochemical screening indicated the presence of flavonoids, alkaloids and saponins in WF. High Performance Liquid Chromatography (HPLC) analysis revealed the presence of hydroxycitric acid and its lactone in ME and WF of G. atroviridis fruit extracts.

CHAPTER ONE

INTRODUCTION

1.1 The plant (Garcinia atroviridis)

1.1.1 Botanical aspects

Scientific name : Garcinia atroviridis Griffith ex T. Anders

Common name : Asam keping or kayu gelugor

Local name : Gelugor (Malay), som khaek (Thailand)

Family : Clusiaceae / Guttiferae

Garcinia genus has been used as medicinal herb for many centuries in tropical region of Asia, Africa and Polynesia (Whitmore, 1973). It was reported beneficial for the treatment of obesity and for control of dietary intake (Amran et al., 2009, Roongpisuthipong et al., 2007). Garcinia atroviridis one of herbs from genus Garcinia is an endemic species in Peninsular Malaysia with a medium-sized fruit tree, which may be found growing wild or cultivated (Mackeen et al., 2002, Permana et al., 2001).

1.1.2 Plant description

G. atroviridis is a medium-sized tree that grows up to 15-20m height (Plate 1.1). It has a long trunk with smooth pale grey bark and drooping branches. The leaves are dark green when mature, glossy, decussate arrangement and narrowly oblong, late tapering to base with a pointed tip and upturned edges. The flowers are dark red. The fruits are globose and around 7-10cm in diameter, pumpkin-like features, borne

singly on twig ends (Plate 1.2). The fruit contains flattened seeds which are 1.5cm long, surrounded by bright orange pulp (arillode). Young green fruits will turn into bright yellow when ripe (Subhadrabandhu, 2001). The morphological characteristics of Garcinia atroviridis spp. have been summarized in Table 1.1.

Table 1.1 Morphological characteristics of G. atroviridis (Te-chato et al., 2000).

Part	**Morphological characteristics**
Flowering (month of the year)	July-September
Latex color	Yellow
Petal colour	Purple
Stigma	Sessile
Stigma surface	Corrugated
Stigma lobe	80% diameter
Stamen mass	Round
Female flower	Staminodes
Pollen viability	3-5%
Fruit shape	Globose
Fruit color	Green
Fruit surface	Wrinkled
Fruit flavor	Sour

Plate 1.1 G. atroviridis tree

Plate 1.2 Fruit of G. atroviridis

1.1.3 Plant habitat and cultivation

G. atroviridis is a native plant of Peninsular Malaysia, Thailand, Myanmar and India. It has been found wild growing as individual trees in the forest of high rainfall areas especially in the northern part of Peninsular Malaysia and southern Thailand and sometimes cultivated as a home garden plant (Subhadrabandhu, 2001, Burkill et al., 1966).

The reproductive behaviour of G. atroviridis was reported to be facultative apomixis which give the advantage to the female plants to produce the progeny successfully even in a male-free population (Pangsuban et al., 2009). It is gynodioecious plant which commonly planted by propagation of germinating seeds. It takes 6-7 years for the tree to attained maximum fruit yield and 11-12 weeks after fruit setting for the fruit to became ripe. Fruits are produced by female trees. However, sometimes hermaphrodite trees also bear few fruits or have no fruit-set (Pangsuban et al., 2009, Pangsuban et al., 2007). It is not yet proof scientifically, but trees that grown from seedling are observed to result in more male than female trees. Therefore, plants of desired sex which can bear fruits within 4-5 years of grafting may be produced by grafting or inarching bud wood of known sex onto seedling (Subhadrabandhu, 2001).

1.1.4 Phytochemical studies on G. atroviridis

Many parts of G. atroviridis have been studied phytochemically and reported to have wide range of compounds (Table 1.2). The compounds isolated belong to xanthone, organic acid, flavonoids and volatile compounds.

Table 1.2 Chemical constituents of G. atroviridis.

Parts of Plant	Chemical constituents
Root	Atrovirinone and Atrovirisidone (Permana et al., 2001)
	Atrovirisidone B, Aringenin and 3'8"-binaringenin (Permana et al., 2005)
	4-methyldroatrovirinone, Morelloflavone, 7-O-β glucopyranoside, Fukugiside and 14-cis-docosenoic acid (Permana et al., 2003)
Stem Bark	Atroviridin (xanthone) (Kosin et al., 1998)
Fruit	(-)-*β*-Caryophyllene, *β*-Caryophyllene alcohol (*β*-Caryolanol), α-humulene, Humulol, Ginsenol (Tan et al., 2012)
	2-(butoxycarbonylmethyl)-3-butoxycarbonyl-2-hydroxy-3-propanolide and 1′,1″-dibutyl methyl hydroxycitrate (Mackeen et al., 2002)
	Flavonoids (myricetin, quercetin & luteolin) (Miean and Mohamed, 2001)
	Organic acids (citric, hydroxycitric, tartaric, malic and ascorbic acid) (Amran et al., 2010, Amran et al., 2009, Zainal Abidin, 2005)

1.1.5 Pharmacological activities of G. atroviridis

G. atroviridis is popularly used as folklore medicine for the treatment of earache, postpartum medication, throat irritation, cough, dandruff, stomachache associated with pregnancy, swollen abdomen and some may mix the fruit extract with vinegar lotion for topical application upon the abdomen of a woman after childbirth (Ahmad, 2010, Grosvenor et al., 1995, Burkill et al., 1996). The pharmacological activities of G. atroviridis have been studied previously are shown on Table 1.3.

Table 1.3 Pharmacological activities of G. atroviridis.

Parts of Plant	Pharmacological activities
Fruit	Improve blood circulation, expectorant, cough, laxative and vasodilatation (Yapwattanaphun et al., 2000)
	Reduce cholesterol in blood and dietary supplement (Amran et al., 2009)
	Treat obesity (Roongpisuthipong et al., 2007)
	Anti-oxidant (Ikram et al., 2009)
	Anti-bacterial and anti-inflammatory (Mackeen et al., 2000)
	Anti-fungal (Mackeen et al., 2002)
	Anti-glycation (Povichit et al., 2010)
Root	Anti-microbial and Anti-oxidant (Mackeen et al., 2000)
Leave	Anti-oxidant (Mackeen et al., 2000)
Trunk	Anti-oxidant (Mackeen et al., 2000)
Stem Bark	Anti-oxidant (Mackeen et al., 2000)

1.2 Inflammation

1.2.1 Overview of Inflammation

The mechanisms for combating the threat of infection and for promoting healing and restoration to the normal function in the event of injury are termed as inflammation. It is the vital function when the body response for protection towards noxious stimuli (chemical, mechanical or pathogens) or a process of reparation, triggered by a complex interrelated cascade system in the body (Spector and Willoughby, 1963). There are five cardinal signs that indicate inflammation: heat (calor), redness (rubor), swelling (tumor), pain (dolor) and loss of function (functio laesa) (Ryan and Majno, 1977). Inflammation involves three important components; harmful stimuli such as pathogen or chemical irritation, sensors such as receptors on mast cells and macrophages, and mediators such as cytokines, histamine (H), prostaglandins (PGs) family and bradykinin (BK). The process of inflammation can be characterized into three phases; increased in vascular permeability that lead to oedema, leukocytes infiltration from blood into the tissues and formation of granuloma (Vogel, 2007; Tripathi, 2008).

1.2.2 Acute and chronic inflammation

Acute inflammation is normally a localized, protective response following trauma or infection, which can be resolved within a short period of time. It usually compromise two components which are innate non-adaptive response and adaptive (acquired or specific) immunological response (Figure 1.1). The innate response is an immediate response activated after infection of injury, comprises of vascular and cellular reaction, meanwhile adaptive immune response is highly specific defensive mechanism that only

starts up after the pathogen has been recognized by the innate system and normally involves the lymphocytes (Rang and Dale, 2007). However, inappropriate control of the defense mechanism during acute response or the persistent of pathogen in the body system may ultimately contributed to the inflammation into chronic stage. This reaction is slow and it could destroy tissues, thus taking longer time to resolve (Kumar et al., 2007).

During acute inflammation, abrupt response mediated by autacoids (e.g. eicosanoids, nitric oxide, kinin, serotonin and histamine) will occur to precedes the development of immunity. Acute response occur in short duration, resolves within hours or days. The immediate response will trigger a variety of inflammatory cells to move to the injured tissues such as polymorphonuclear leukocytes (PMNs) (e.g. neutrophils, basophils and eosinophils) which are attracted by the chemoattractants released by the injured cells. Initially, phagocytic leukocytes known as neutrophils which prevalently available during acute inflammation will migrate to the inflamed site and followed by the accumulation of monocytes which then activated into macrophages (Serhan, 2008). Activated macrophages will synthesize varieties of bioactive lipids such as eicosanoids (prostaglandin and leukotrienes) and platelet activating factor (PAF), and also release pro-inflammatory cytokines notably as tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) (Laskin and Pendino, 1995).

The ability of the body to act briefly and rapidly towards the clearance of harmful stimuli by interconnected inflammatory mechanisms are very important as to avoid overwhelming situations. Persisted and the presence of long-lasting injury causing agent can be detrimental to the host. Acute inflammation can become chronic if the normal

healing process in acute inflammatory response is unsuccessful to ingest the toxic injuring causing material. Previous studies have reported, in uncontrollable situation, acute inflammation could turn into chronic condition by the powerful influences of most pro-inflammatory mediators such as prostaglandins and leukotrienes and, various cytokines such as TNF-α, IL-1 and chemotactic cytokines (Langhans, 2006, Lawrence et al., 2002, Feghali and Wright, 1997).

Primary chronic inflammation can occur if the responsible agent is unclear. It is the subsequent tissues reaction towards injury. The inflammation process may become exaggerated, in which often associated with the accumulation of lymphocytes, plasma cells and formation of granuloma from the fusion of multinucleated gigantic cells (MGC) (Kunkel et al., 1989). Multinucleated gigantic cells are well known to form from the fusion of the phagocytic leukocytes (macrophage) and plays important roles in physiological and pathological function (Helming and Gordon, 2007; Vignery, 2008). In healthy human, MGC are known as osteoclasts which are found in bone, responsible for bone resorption (Vaananen et al., 2000). However, as a results of chronic inflammation, giant cells can arise in the nonskeletal tissues due to the presence of foreign materials that are poorly digestible or persistent pathogens which have not been killed yet. The multinucleated macrophage involves in the clearance of internalized particles, apoptotic cells and debris (Ruibal et al., 1997).

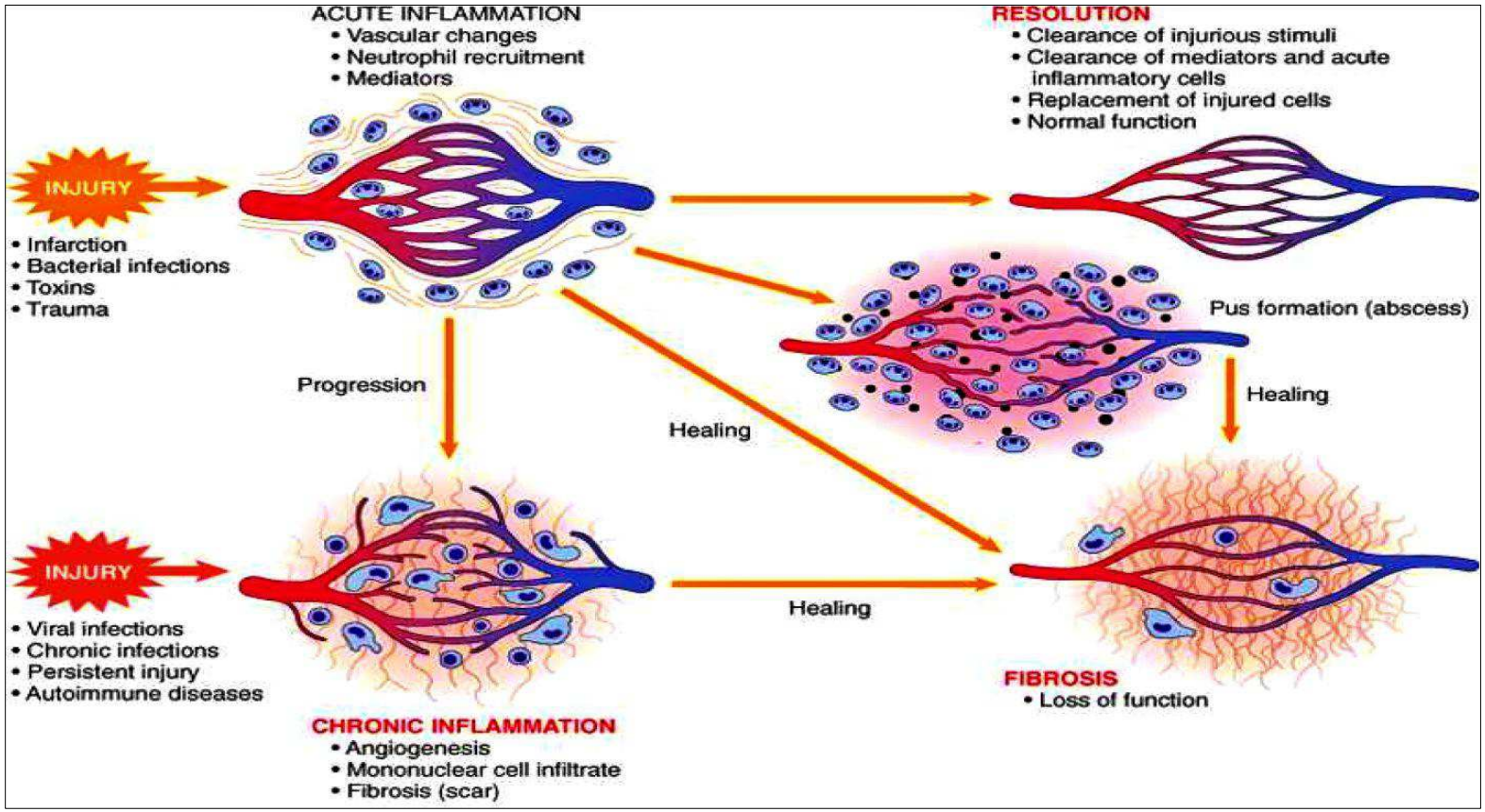

Figure 1.1 Acute and chronic inflammation (Kumar et al., 2007).

1.2.3 Mediators in inflammation

The complex pathophysiological systems in the response towards injury are controlled by various inflammatory mediators and inflammatory cytokines, involved both in acute and chronic inflammation. Further details on the roles and mechanisms involved are discussed below.

i) Histamine

As reported by Mans et al., (2008), histamine was first discovered as H substance by Dale and Laidlaw in 1910. In the preparations of isolated guinea pig ileum, it has shown the capability of producing the contraction of smooth muscle. This biogenic amine is stored in granules of mast cells and basophils. It was reported that the composition of an imidazole ring with an ethylamine side chain on the histamine had mediated both physiologic and pathologic roles played by it (Mans et al., 2008).

Several studies have documented the implication of histamine on immune regulation which mediated by differential expression of histamine receptors (H_1R, H_2R, H_3R and H_4R) through various stimuli, which suggested the important roles of histamine biologically and pharmacologically (Parson and Ganellin, 2006; MacGlashan, 2003; Jutel et al., 2002; Schneider et al., 2002). The actions of histamine was reported to be depending on which receptors were being activated. The expression of H_1 receptor were reported to increase in in vitro differentiation of monocytes into macrophages (Triggiani et al., 2007), while the expression of H_4 receptors in human monocytes together with its stimulation had lead to the influx of calcium ion, results in the reduction of monocytes recruitment (Dijkstra et al., 2007).

In the early phase of inflammation, the vasoactive amine such as histamine is the major vasodilator in controlling the vascular activity (Crunkhorn and Meacock, 1971; Di Rosa et al., 1971). Histamine has been reported to produce pain and three major 'vasoactive' effects; transient vasoconstriction which followed by vascular dilatation of arterioles, capillaries, and venules; fluctuation in blood flow (increased to decrease); and finally venular leakage which results in the extravasations of plasma protein due to the endothelial contraction (Majno and Joris, 2004).

ii) Serotonin [5-hydroxyptryptamine (5-HT)]

Serotonin (5-HT), is a copious neurotransmitter and neuromodulator component of inflammatory chemical milieu. It is normally found in normal cells that are not neurons and stored in granules of platelets, mast cells, central nervous system and the enteric neural tissues, before infiltrate the tissue damage area (Dray, 1995). Once released, the contribution of it to the pain injury is through the interaction of multiple receptor subtypes expressed by primary afferent nociceptors (Zeits et al., 2002; Pauwels, 2000).

5-HT is one of the first mediators reported to be released after injury, and seems did not play any role in chronic inflammation (Majno and Joris, 2004; Di Rosa et al., 1971). As implied by the name "serotonin", serum substances that increase pressure, similar with histamine, the vasoactive properties in serotonin increased the vascular permeability with a greater tendency in inducing the contraction of the vessels (Majno and Joris, 2004).

The role of 5-HT as pro-inflammatory mediators have been reported previously by numerous studies of inflammation and pain. The released of 5-HT to the inflamed site

as pro-inflammatory and pro-nociceptive, excite the nociceptive afferents, thus inducing pain and hyperalgesia (Sommer, 2004; Schmelz et al., 2003; Ernberg et al., 2000; Kessler et al., 1992; Sufka et al., 1992; Taiwo and Levine, 1992). The mechanisms behind pain and hyperalgesia induce by 5-HT are determined by the multiple 5-HT receptors on the afferent nociceptors. Previous study on the formalin-induced nociceptive behaviours and oedema had revealed the involvement of 5-HT_1 and 5-HT_3 receptors in the peripheral nociception associated with acute inflammation and oedema formation (Doak and Sawynok, 1997).

5-HT plays important roles in immune system. It acts as activator of the human monocytes and as a preventer of apoptotic activity (Soga et al., 2007). Through multiple 5-HT receptors subtypes, it also act as a modulator of the cytokines and chemokines production in LPS-primed monocytes (Dürk et al., 2005). In addition, a study on the experimental serum sickness in rabbit found that, antagonizing the activity of 5-HT have prevent the increased in vascular permeability, thus inhibit the localization of immune complexes (Kniker and Cochrane, 1968).

iii) Kinin-bradykinin system

The kinin-bradykinin system is composed of the precursor of kinin; kininogens, which could be activated by plasma and tissue kallikreins, thus producing two vasoactive nonapeptide namely bradykinin (BK) and kallidin. In humans, there are two kininogens known as high molecular weight (HMW) and low molecular weight (LMW) kininogens which could be converted into bradykinin. Meanwhile, in rats, in addition to the HMW and LMW, there is another precursor known as T-kininogen (Oh-ishi et al., 1987; Okamoto and Greembaum, 1983).

This system is mediated through two receptors noted as B_1 and B_2 (Regoli et al., 1993). The activation of this system is important as it regulates physiological and pathological function such as blood pressure and inflammatory response due to its roles as vasodilator, which could induced oedema formation, cellular accumulation and pain mostly mediated by B_2 receptors (Shin et al., 2002, Agostoni and Cugno, 2001, Lewis, 1958). In addition, the B_1 receptors are also reported to be involved at the chronic inflammation by amplifying the kinin B_2 receptors (Ahluwalia and Perretti, 1999).

During inflammation, the kinins system acts as pro-inflammatory mediators as it could stimulate the synthesis of prostaglandins families (PGs and PGI_2), leukotrienes, histamine, cytokines and platelet activating factor (PAF), thus promoting the inflammatory process (Stadnicki et al., 2005; Couture et al., 2001, Sharma and Mohsin, 1990). The upregulation of both kinins B_1 and B_2 receptors on neutrophils and macrophages appears to involve the elevation of local release of bradykinin at the site of inflammation. The stimulation of both receptors in macrophages induce production of various cytokines and inflammatory mediators (Böckmann and Paegelow, 2000).

iv) Prostaglandins (PGs)

Prostaglandins are lipid autocoids, or collectively called eicosanoids, generated from arachidonate, and universally distributed in the body. The cyclic eicosanoids play important roles, depend on the types of PGs, some involve in physiological maintenance by sustaining homeostatic function (act as autocrine and paracrine lipid mediators) and others involve in pathophysiological activity during inflammatory response (Tripathi, 2008).

In uninflammed tissues, PGs production is maintained at lower level. However, upon tissue injury, dramatical change in the prostanoids profiles and increase in the levels of PGs could occurred, which contributes to the development of cardinal signs of acute inflammation prior to the emigration of leukocytes from the vessels. The infiltration of immune cells (e.g. leukocytes) result in more prostanoids being released at the area on inflamed tissues, thus involve more complex immune system function (Ricciotti and FitzGerald, 2011).

Arachidonic acid is metabolized by sequential actions of prostaglandin endoperoxide (PGG/H) synthase or colloquially known as COX, bifunctional enzymes (COX-1 and COX-2) which resulted in four series of PGs, namely, prostaglandin E_2 (PGE_2), prostacylin (PGI_2), Prostaglandin D_2 (PGD_2) and prostaglandin F2α (PGF2α) (Ricciotti and FitzGerald, 2011). Further details on the roles played by each prostanoid are explained below.

i) PGE_2

PGE_2 is one of the prostanoid that elicits a wide range of biological and pathophysiological functions. It regulates the immune responses, blood pressure, gastrointestinal integrity and fertility. It also contributes to classical signs of acute inflammation, which are redness and swelling that result from the increased in microvascular permeability due to the PGE_2 augmentation of arterial dilatation and pain results from the action of PGE_2 on the peripheral sensory neurons and on central sites within the spinal cord and the brain (Moriyama et al., 2005; Funk, 2001; Minami et al., 2001).

PGE_2 is synthesized from PGH_2 by cytosolic prostaglandin E_2 synthase (cPGES), which constitutively and abundantly expressed in cytosol of various tissues and cells and also synthesized by membrane prostaglandin E synthase-1 (mPGES-1), a perinuclear protein involved in eicosanoid and glutathione metabolism. The ability of both cPGES and mPGES-1 to form PGE_2 require glutathione as cofactor. cPGES is only capable to convert COX-1 derived PGH_2 to PGE_2 in cells, particularly the inflammatory response evoke by Ca^{2+} stimuli (Samuelsson et al., 2007; Tanioka et al., 2000). Meanwhile, mPGES-1 is markedly induced by cytokines and growth factor, and functional coupling with COX-2 in marked preferences to COX-1 (Mancini et al., 2001, Murakami et al., 2000, Thorén and Jakobsson, 2000).

The PGE_2 is transported through the membrane by the ATP-dependent multidrug resistance protein-4 or diffused across the plasma membrane (Park et al., 2006) and act locally through the binding of its cognate receptors, known as EP_1, EP_2, EP_3 and EP_4 (Trebino et al., 2003). Highest affinity for binding of PGE_2 goes to EP_3 and EP_4 which found to be widely distributed in the system, compared to EP_1 that only available in restricted organs and EP_2 be the least abundant of the EP receptors (Sugimoto and Narumiya, 2007).

Previous study on Knockout (KO) mouse has reported the role of PGE_2 as pro-inflammatory and anti-inflammatory mediators which regulated by the EP_1 receptor signalling that acts on peripheral sensory neurons at the site of inflammation. The four cardinal signs during acute inflammation and hyperalgesia also found to be mediated through this mechanism (Moriyama et al., 2005). On the other hands, other study has

reported that pain response was mediated by the EP_3 receptor signaling at low doses of PGE_2 (Minami et al., 2001).

The ability of PGE_2 to bind to different EP receptors allow the inflammatory mediator to modulate the functions of macrophages, dendritic cells (DC) and T and B lymphocytes which then lead the dual role of PGE_2 during inflammation process, as the coordinator of both pro-inflammatory and anti-inflammatory effects (Ricciotti and FitzGerald, 2011). The action of PGE_2-EP_4 signaling as pro inflammatory was reported previously through the regulation in the cytokine expression of DCs which facilitated the helper T (T_H) receptor, T_H1 differentiation (Yao et al., 2009). In addition, stimulation of PGE_2 during early maturation of DCs had induced the expression of TNF super family resulted in the enhancement of the T cell activation (Krause et al., 2009).

In contrast, PGE_2 as anti-inflammatory was found in the previous study demonstrated by the roles of the prostanoids in suppressing the Th1 differentiation, B cell functions and allergic reactions. Besides that, PGE_2 also can exert the anti-inflammatory properties on innate cells, such as neutrophils, monocytes, and natural killer cells (Harris et al., 2002).

Previous study on atherosclerosis also revealed the dual roles played by PGE_2, which not only promotes apoptotic activity of the macrophage, it also reported to suppressed early atherosclerosis in mice chimeric for EP_4 receptor deficiency in hematopoietic cells after 8 weeks on Western style diets containing 21% milk fat and 0.15% cholesterol. In the same study, PGE_2-EP_4 signaling of the macrophage reported to act as pro-inflammatory mediators by regulating the production of inflammatory cytokines such as IL-1β and IL-

6 and monocyte chemotactic protein-1 at the early stages of atherosclerosis (Babaev et al., 2008).

ii) PGI_2

PGI_2 is prostanoid that regulates homeostasis process in the body especially the cardiovascular-related activity. It is generated through the sequential actions of COX and PGI_2 synthase (PGIS), a cytochrome P450 superfamily specific in converting PGH_2 to PGI_2 (Smith et al., 1983). It is a potent vasodilator and act as inhibitor on the circulating platelets, leukocytes adhesion and proliferation of vascular smooth muscle cells (Ricciotti and Fitzgerald, 2011), mediated by specific prostanoid receptor (IP) which expresses in kidney, liver, lung, platelets, heart and aorta (Smyth and FitzGerald, 2002).

PGI_2 was also reported to be an important mediators of oedema and pain in acute inflammation. This prostanoid was found abundantly in the synovial fluid in human arthritic knee joints and peritoneal cavity fluid of mice injected with irritants such as acetic acid, phenylbenzoquinone and zymosan (Berkenkopf and Weichman, 1988; Higgs et al., 1983). In the case of PGI_2 actions in the pain that accompany acute inflammation, previous studies have reported the role of the prostacylin receptor (IP receptor) mRNA, which could be present in dorsal root ganglion neurons (Oida et al., 1995) and at the spinal cord (Doi et al., 2002). IP receptor antagonists (e.g. RO1138452 and RO3244794) were shown to reduce pain responses by mediating the peripheral nociception sensitized by inflammatory stimuli such as acetic acid-induced writhing and pain responses in carrageenan-induced hyperalgesia and inflammatory arthritis (Bley et al., 2006, Pulichino et al., 2006, Murata et al., 1997).

iii) $PGF_{2\alpha}$

$PGF_{2\alpha}$ is synthesized from PGH_2 via PGF synthase, and was reported to be stable metabolite in the form of 15-keto-dihydro-$PGF_{2\alpha}$ that could be found in the peripheral plasma and urine in both physiological and pathophysiological conditions such as inflammation (Basu et al., 2007). $PGF_{2\alpha}$ has been reported to play important roles in the arteries contraction (Nakahata et al., 2006), myocadial dysfunction (Jovanović et al., 2006) and pain (Kunori et al., 2009). Previous in vivo and in vitro study reported that administration of $PGF_{2\alpha}$ caused acute inflammation but the biosynthesis was inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) (Sugimoto et al., 1997). Patients suffered from various arthritis disease such as rheumatoid arthritis and osteoarthritis were found to have elevated synthesis of this prostanoids (Basu et al, 2001).

1.2.4 Cytokines in inflammation

Cytokines are a group of secreted polypeptide derived from different types of cells, which involved in extensive networks in immune response, and categorized as pleiotropic molecules due to their functionality to elicit either local or systemic response in an autocrine or paracrine, rather than endocrine manner (Balkwill and Burke, 1989). This group can be classified into cytokines mediated by humoral responses such as IL-4, IL-5, IL-6, IL-7, IL-13 and cytokines mediated by cellular responses such as IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-10, IL-12, tumor necrosis factor α and β, interferons and transforming growth factor-β (Feghali and Wright, 1997).

Became the major determinants that make up the cellular infiltration and activation which then lead to the systemic response towards inflammation, cytokines involvement

during inflammation were widely studied. Some cytokines may act synergistically or antagonistically and some may have multiple overlapping biological functions (Paludan, 1998; Le and Vilcek, 1987). The presence of cytokines virtually undetectable in normal condition except under pathological condition.

Inflammation that occurred in cutaneous tissue was showed to be involved a distinct cytokine cascade. TNF and IL-1α and β have been extensively reported to be the primary cytokines involved during both acute and chronic inflammation (Alciato et al., 2010; Yuan et al., 2010; Brooks et al., 1996; Semenzato, 1990; Hart et al., 1989). Upon injury, bradykinin that derived from the mast cells will stimulate the release of tumor necrosis factor alpha (TNF-α), which will subsequently stimulates the released of interlekuin-1 beta (IL-1β) and interlekuin-6 (IL-6), which in turn will promote the released of cyclooxygenase enzymes that play a major role in the prostaglandin biosynthesis (Poole et al., 1999). It was reported previously that during inflammation, IL-1β had played a major role as cytokine stimulus for central COX-2 expression (Samad et al., 2001).

Besides that, IL-1β could be induced by both TNF-α dependent and TNF-α independent pathways (Woolf et al., 1997). Both TNF-α and IL-1 act as pro-inflammatory to promote fever by eliciting the release of histamine from the mast cells, thus increasing vasodilatation and vascular permeability or the action might also through the stimulation of PGE_2 synthesis of the vascular endothelial of the hypothalamus (Dinarello et al., 1986). As mentioned earlier, the production of cytokines was also regulated by other pro inflammatory mediators. Previous observation on the secretion of TNF-α and IL-1β from

monocyte-derived dendritic cells were found to be regulated by 5-HT through the stimulation of 5HTR-4 and 5HTR7 (Idzko et al., 2004).

1.3 Cyclooxygenases (COX-1 and COX-2) and inflammation

Cyclooxygenase (COX) or also known as prostaglandin endoperoxide synthases is enzyme that catalyzes the conversion of arachidonic acid to PGG_2 to PGH_2 by peroxidase activity. The enzyme catalysis is the initial step in the biosynthesis of PGs, thromboxanes and prostacyclin (Tripathi, 2008, Hamberg et al., 1975).

COX is known to exist in two isoforms; COX-1 and COX-2. While both have the same active site and catalyze the same reactions, COX-1 is a constitutive isoform that involve in normal cellular physiologic function that lead to the production of prostacyclin which when released by gastric mucosa is cytoprotective (Whittle et al., 1980). On the other hand, COX-2 is inducible isoform which when it is induced by mitogenic stimuli (lipopolysaccharide, phorbol myristate acetate, cytokines) and it would plays major role in the biosynthesis of PGs during acute inflammation (Vane DSc et al., 1998, Kujubu et al., 1991, Xie et al., 1991).

Both COX isoforms are involve in acute inflammatory response, even only COX-2 was found to be the dominant source of PG during the event of inflammation. Previous studies have reported the co-expression of both COX isoforms in the synovium and atherosclerotic plaque obtained from patients with rheumatoid arthritis (RA) and also from the circulating inflammatory cells ex vivo (Schönbeck et al., 1999; Crofford et al., 1994).

Several findings have indicated the contribution of both isomers in the PGs production. While COX-1 isoform has been reported to be constitutively expressed in the resident inflammatory cells such as in LPS-mediated inflammatory response and in cellular differentiation (McAdam et al., 2000), COX-2-derived PGs on the other hand play dual roles in inflammatory process in the onset of inflammation and in the resolution process. The resultant inflammation observed in the paw oedema induced by carrageenan has been reported to be resolved within 7 days in WT mice but not in COX-2-deficient mice (Wallace et al., 2000).

In another study on carrageenan-induced pleurisy in rats, the COX-2 expression were reported to be peaked initially at early inflammatory response, whereas COX-1 expression remained constant. PGE_2 was reported to increase to high level during this early phase. However, a second increase of COX-2 expression to a greater level was reported at later phase. This mechanism was reported to be associated with the low synthesis of PGE_2, but with the greater generation of PGD_2 and 15deoxyΔ^{12-14} prostaglandin J_2 (5deoxy$\Delta^{12-14}PGJ_2$). Thus, from the study, it was indicated that, COX-2 act as pro-inflammatory during the early inflammatory response, which reported to be dominated by the polymorphonuclear leukocytes (PMNL) for the first 12 hours, while it acts as anti-inflammatory during the resolution phase at 48 hours, which reported to be dominated by the migrating mononuclear (MN) cells that differentiated into macrophages (Gilroy et al., 1999).

The inflammatory stimulus and experimental procedure used determined which COX isoforms responsible for the inflammation. In a case of ear inflammation studies, the oedema induced by tetradecanoyl phorbol acetate (TPA) in COX-1 and COX-2 deficient

mice were not significantly different. In contrast, a reduction in oedema induced by arachidonic acid was only found in COX-1-deficient mice, but not in COX-2 deficient-mice (Dinchuk et al., 1995; Langenbach et al., 1995; Morham et al., 1995).

As in different experimental model being studied, COX-1-derived PGs, particularly PGI_2 was found to be the major contributor in the initiation and perpetuation of arthritis in the K/BxN serum-transfer model (Chen et al., 2008). Meanwhile, the suppression of the synovial inflammation and joint destruction in the collagen-induced arthritis model were found to be significant due to COX-2 deletion, rather than the indistinguishable suppression showed by the COX-1-deficient-mice when compared to the controls (Ochi et al., 2003; Myers et al., 2000).

1.4 Non-Steroidal Anti-inflammatory Drugs (NSAIDs)

In the early of 1970s, it was proposed that the production of prostaglandin was through the inhibition of the cyclooxygenase enzymes, suggesting the mechanism action of non-steroidal anti-inflammatory drugs (NSAIDs), by blocking the PGs generation (Smith and Willis, 1971; Vane, 1971). Several other drugs with similar therapeutic effects as being anti-pyretic, anti-inflammatory and analgesic like aspirin, such as paracetamol, indomethacin, ibuprofen and naproxen become known as "aspirin-like drugs", and due to distinct mechanisms of action from glucocorticosteroids, they were also classified as NSAIDs (Flower, 1974). They are non-narcotic and do not depress the central nervous system, no dependency and are weaker analgesics (Tripathi, 2008).

Unlike glucocorticosteroids that inhibit the release of arachidonic acid from the membrane phospholipid which then indirectly reduced the production of eicosanoids such as prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs), NSAIDs in the other hands only inhibit cyclooxygenase (COX) pathway, production of TXs and prostacylin, but not lipoxygenase (LOX) pathway, the main pathway producing leukotrienes (Figure 1.2). NSAIDs posses numerous therapeutic indications recognized as being anti-inflammatory by reducing redness and swelling; anti-pyretic by reducing high temperature and analgesic by alleviating pain (Tripathi, 2008).

The major mechanism of action of NSAIDs is corresponds to their potency to inhibit COX pathway, thus inhibit prostaglandin biosynthesis. NSAIDs such as aspirin, paracetamol and ibuprofen could treat acute condition such as postoperative pain, fever and relieve the symptoms of upper respiratory tract infection (URTI) (Derry and Moore, 2012; Bachert et al., 2005; Collins et al, 2000). In addition, most NSAIDs such as celecoxib, diclofenac, ibuprofen, ketoprofen and peroxicam generally approved to treat chronic health problem such as osteoarthritis and rheumatoid arthritis (Altman and Barthel, 2011; Zhang et al., 2008; Clemett and Goa, 2000).

NSAIDs was reported previously to be effective in inflammation associated pain. However, prolong used of potent COX-1 inhibition such as aspirin, indomethacin and peroxicam for treatment had caused gastric mucosal damage, thus results in ulceration (Lanza, 1989). NSAIDs with higher selectivity against COX-2 are more favorable for the anti-inflammatory activity as it caused fewer side effects to the mucosal lining of stomach (Rodriguez and Jick, 1994). However, recent studies have also reported the used of COX-2 inhibitor had reduced the vascular prostacylin synthesis, thus