# CHAPTER 1
# INTRODUCTION

## 1.1 Statement and significance of the problem

In the present daily life, human must defend itself against multitude of different pathogens including viruses, bacteria, fungi, protozoan and metazoan parasites as well as tumors and a number of various harmful agents which are capable to derange its homeostasis. For this purpose, plenty of effector mechanisms capable of defending the body against such antigens and agents have developed and these can be mediated by soluble molecules or by cells. If infection occurs as a consequence of the tissue damage, the innate and later, the adaptive immune systems are triggered to destroy the infectious agent. Inflammation occurs as a consequence of defensive response intended to eliminate the initial cause of cell injury as well as the necrotic cell and tissue resulting from the original insult. It is commonly associated with several symptoms or diseases, such as pain, fever, rheumatoid arthritis (RA), psoriatic arthritis, osteoarthritis (OA), gout, etc. Drugs for treatment of patients with inflammatory diseases can be divided into non-steroidal anti-inflammatory drugs (NSAIDs), and anti-inflammatory corticosteroids. NSAIDs are among the most commonly used drugs worldwide and are an important class of drugs used to treat inflammatory conditions. NSAIDs are used to treat pain and inflammation in a variety of conditions and produce their effect by inhibition of cyclooxygenase (COX). A major drawback of NSAID use is the high incidence of gastrointestinal side effects, which lead to the development of the selective COX-2 inhibitors. Consequently, the side effects of anti-inflammatory drugs are one of the major problems in developing medicine today (Kayaalp, 1998, Rampasath *et al*., 2004). In the recent years, a wide-spread search has been launched to identify new anti-inflammatory drugs from synthetic and natural resources. It has been reported that many plants with anti-inflammatory activity have been found to lack an ulcerogenic effect. For examples, *Curcuma longa* Linn. (Yegnanarayan *et al*., 1976; Rafatullah *et al*., 1990), *Pluchea indica* Linn. (Sen and Nag Chaudhuri, 1991; Sen, *et al*., 1993), *Turnera ulmifolia*

(Antonio and Souza Brito, 1998; Gracioso *et al*., 2002), and *Zingiber officinale* Roscoe (Suekawa *et al*., 1986; Yamahara *et al*., 1988; Yoshikawa *et al*., 1994). Euphorbiaceae is one of the very interesting family. Many extracts or fractions from plants of this family have been reported to possess anti-inflammatory activity. These include the extract of *Tragia involucrata* Linn. (Dhara *et al*., 2000; Samy *et al*., 2006), the aqueous extract of *Bridelia ferruginea* stem bark (Olajide *et al*., 2000), the ethyl acetone stem bark extract of *Bridelia scleroneura* (Theophile *et al*., 2006), the aqueous extract of *Euphorbia hirta* Linn. (Lanhers *et al*., 1991), and the leaf extract from *Mallotus peltatus* (Chattopadhyay *et al*., 2002). Moreover, *Phyllanthus amarus* (Kassuya *et al*., 2003, 2005) and *P. polyphyllus* (Rao *et al*., 2006) also show anti-inflammatory and analgesic activities. Therefore, it is of interest to prove such effects of *Phyllanthus emblica* Linn., which also belongs to this family.

Herbal medicines are popular and extensively used in the developing world. In many places, they offer a readily available and more affordable alternatives to pharmaceutical drugs. The World Health Organization (WHO) estimates that a large proportion of the world's population relies heavily on traditional practitioners and medicinal plants in order to meet primary health care need (WHO, 1999). One-third of the world's population and up to half of the populations in the poorest parts of Asia and Africa do not have access to essential drugs (WHO, 2001). For these populations, traditional medicine presents a particularly promising opportunity to bridge the gap between those who need health care and those who provide health services. The WHO has passed a number of resolutions in response to a resurgence of interest in the study and use of traditional medicines in health care, and in recognition of the importance of medicinal plants to the health systems of many developing countries. In Thailand, most of medicinal plants are collected from the forests or natural sources. These plants are commonly used as tea, tincture or filtrate. Since these methods are inexpensive and can be performed with a minimal amount of training, herbal medicine holds great promise for use in a primary health care environment. Safety and efficacy data are available for an even smaller number of plants, their extracts and active ingredients and preparations containing them. In recent years the Thai government has had a policy to develop medicinal plants knowledge to provide remedies for many diseases, such as gastrointestinal problems, diseases of upper

respiratory tract, urinary tract diseases and skin diseases. Many hospitals in Thailand use herbal medicines to treat patients; this is not only beneficial to the health care as a whole but also to the economy of the country. Thus, herbal medicines are the particular value in alternative health care for Thai people. Therefore, medicinal plants in Thailand should be rigorously identified and investigated for their pharmacological activities, toxicity study should also be carried out.

## 1.2 Literature review

### Inflammation

Inflammation is the body's reaction to invasion by an infectious agent, antigen challenge or even just physical, chemical or traumatic damage. Moreover, inflammation can be defined as the tissue change in reaction to injury. These changes characteristically involve vascular and cellular responses working together in a coordinated manner to destroy substance recognized as being foreign to the body (Guyton and Hall, 2000). The five cardinal signs of inflammation are redness, swelling, heat, pain and deranged function. These signs are due to extravasation of plasma and infiltration of leukocytes into the site of inflammation. The main features of the inflammatory response are vasodilatation, i.e. widening of the blood vessels to increase the blood flow to the infected area; increased vascular permeability, which allows diffusible components to enter the site; cellular infiltration by chemotaxis, or the directed movement of inflammatory cells through the walls of blood vessels into the site of injury; changes in biosynthetic, metabolic, and catabolic profiles of many organs; and activation of cells of the immune system as well as of complex enzymatic systems of blood plasma. Of course, the degree to which these occur is normally proportional to the severity of the injury and the extent of infection.

Inflammatory response occur in three distinct phases, each apparently mediated by different mechanism: (1) an acute transient phase, characterized by local vasodilatation and increase vascular permeability; (2) a delayed subacute phase, most prominently characterized by infiltration of leukocytes and phagocyte cells; and (3) a chronic proliferative phase, in which tissue degeneration and fibrosis occur. Many different mechanisms are involved in the inflammatory process (Robert II and Morrow, 2001).

Acute inflammation is short-lasting process occurring only a few days. The acute inflammatory response consists of 3 main characteristics.

1) The affected area is occupied by a transient material called the acute inflammatory exudate. The exudate carries proteins, fluid and cells from local blood vessels into the damaged area to mediate local defenses.
2) If an infective causitive agent (e.g. bacteria) is present in the damaged area, it can be destroyed and eliminated by components of the exudate.
3) The damaged tissue can be broken down and partialy liquefied, and the debris removed from the site of damage (Stevens and Lowe, 2000).

Moreover, the inflammatory responses consist of changes in blood flow, increased permeability of blood vessels and escape of cells from the blood into the tissues. It is mediated by the release of autacoids such as histamine, serotonin, bradykinin, prostaglandin (PG) and leukotriene (LT). The vascular and exudative phenomena of acute inflammation are responsible for the clinical features (Cree, 1997). Microscopically, it involves a complex series of events including dilation of arterioles, capillaries and venules with increased permeability and blood flow; structural changes in the microvasculature that permit the plasma proteins and leukocytes to leave the circulation and leukocytic migration from the microcirculation and their accumulation into the inflammatory focus (Collins, 1999; Gallin and Snyderman, 1999). The major local manifestations of acute inflammation are shown in Figure 1.

Chronic inflammation is an inflammatory response of prolonged duration - weeks, months, or even indefinitely - whose extended time course is provoked by persistence of the causative stimulus to inflammation in the tissue. Chronic inflammation is mediated by both immunological and nonimmunological mechanism and is frequently observed in conjunction with reparative responses, namely, granulation tissue and fibrosis (Fantone and Ward, 1999). Chronic inflammation is characterized by (1) infiltration with mononuclear cells, which include macrophages, lymphocytes, and plasma cells, a reflection of a persistent reaction to injury; (2) tissue destruction, largely induced by the inflammatory cells; and (3) attempts at healing by connective tissue replacement of damaged tissue, accomplished by proliferation of small blood vessels (angiogenesis) and in particular, fibrosis (Collins, 1999).

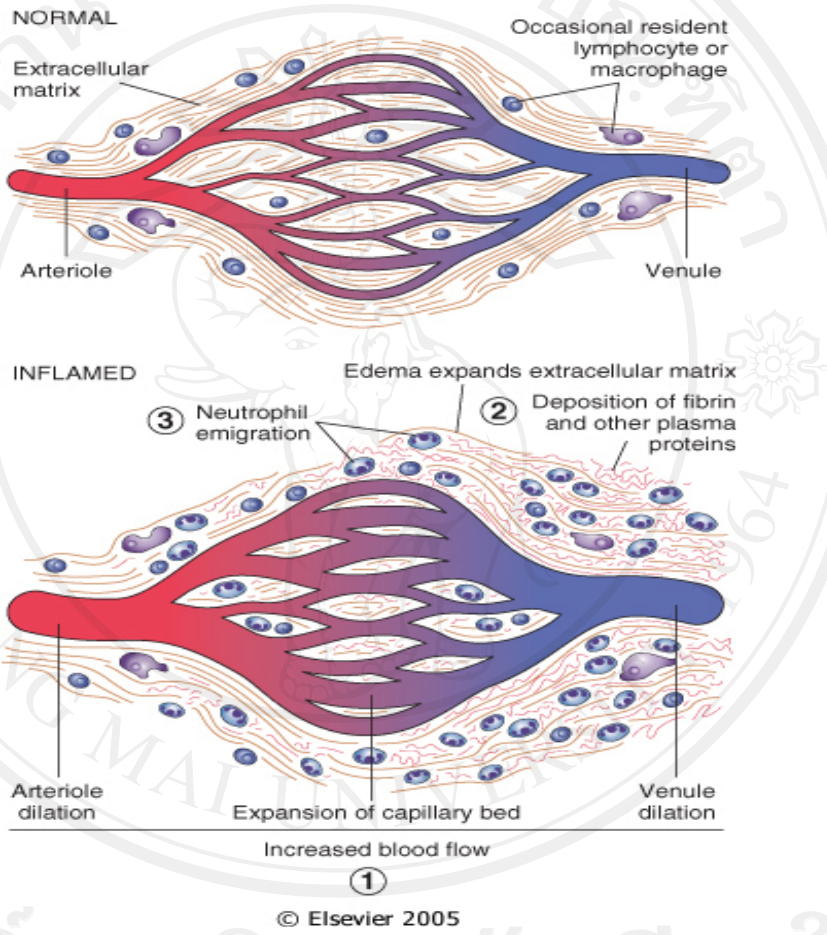


**Figure 1** The major local manifestations of acute inflammation, compared to normal.

Moreover, chronic inflammation involves the release of a number of mediators that are not prominent in the acute response. Some of these are interleukins (IL) 1, 2, and 3, tumor necrosis factor alpha (TNF-α), interferon (IFN) and platelet-derived growth factor (PDGF). This process is initiated by T lymphocytes, then cellular infiltration with T-cells, B-cells, macrophages and plasma cells and subsequent cytokine production (Waller *et al.*, 2005b; Furst and Robert, 2007).

**Inflammatory mediators**

Inflammatory mediators are soluble, diffusible molecules that act locally at the site of tissue damage and infection, and at more distant sites. The mediators derived from arachidonic acid (AA) metabolites are one important group of mediators for inflammation. AA is released from membrane phospholipids through the activation of cellular phospholipases such as phospholipase $A_2$ by mechanical, chemical or physical stimuli. AA metabolites, also called eicosanoids, are synthesized by two major classes of enzymes, COX and lipoxygenase (LOX). Eicosanoids can mediate virtually every step of inflammation and it can be found in inflammatory exudates, and their synthesis is increased at sites of inflammation (Collins, 1999). The COX pathway, mediated by two different enzymes (COX-1 and COX-2), lead to the generation of PGs. Comparison of the property of COX-1 and COX-2 is present in Table 1.

The most important mediators in inflammation are $PGE_2$, $PGD_2$, $PGF_{2\alpha}$, $PGI_2$ (prostacyclin), and thromboxane $A_2$ ($TXA_2$), each of which is derived by the action of a specific enzyme (Collins, 1999). Each of the PGs and $TXA_2$ has a unique profile of actions on blood vessels, smooth muscles, platelets, and other cells. $PGE_2$ and $PGI_2$ decrease tone and increase the permeability of vessels in the microcirculation. In contrast, $TXA_2$ and $PGF_{2\alpha}$ increase microvascular tone and $PGF_{2\alpha}$ slightly decreases the natural permeability of microcirculature as well as the increased permeability induced by other agonists (Collins, 1999, Griffiths, 1999; Serhan, 2001). Moreover, PGs induce a variety of inflammatory effects including fever, edema, erythema, polymorphonuclear chemotaxis, increased vascular permeability, stimulation of bone resorption, and decrease synthesis of type I procollagen (Furst and Hillson, 2001).

**Table 1** Comparison of the properties of COX-1 and COX-2

| Property | COX-1 | COX-2 |
|---|---|---|
| Expression | Constitutive | Inducible; not normally present in most tissues |
| Tissue location | Ubiquitous expression | Constitutive in part of nervous system |
| Cellular localization | Endoplasmic reticulum (ER) | Inflamed and activated tissues, ER and nuclear membrane |
| Substrate selectivity | AA, eicosapentaenoic acids | AA, γ-linolenate, α-linolenate, linoleate, eicosapentaenoic acids |
| Role | Protection and maintenance function | Pro-inflammatory and mitogenic functions |
| Induction | Generally no induction, human chorionic gonadotropin (hCG) can up-regulate COX-1 in amnion | Induced by bacterial lipopolysaccharide (LPS), TNF-α, IL-1, IL-2, epidermal growth factor (EGF), IFN-γ |
| Inhibition | *In vivo*: Anti-inflammatory glucocorticoids. Pharmacologic: Non-steroidal anti-inflammatory drugs (NSAIDs) | *In vivo*: Anti-inflammatory glucocorticoids. Pharmacologic: NSAIDs, COX-2 selective inhibitor |

The LOX pathway of AA metabolism produces LTs, these are also involved in the inflammatory process by enhancing vascular permeability and through chemotactic attraction of leukocytes (Waller *et al*., 2005a). The initial products of the LOX pathway are generated by three different lipoxygenases, which are present in only a few types of cells; 5-lipoxygenase (5-LOX) is the predominant enzyme in neutrophils. The main product is 5-hydroxyl fatty acid (5-HETE), which is chemotactic for neutrophils, is converted into a family of compounds collectively call leukotrienes ($LTB_4$, $LTC_4$, $LTD_4$ and $LTE_4$). $LTB_4$ is a potent chemotactic agent and activator of neutrophils functional responses such as aggregation and adhesion of leukocytes to venular endothelium, generation of oxygen free redicals, and release of lysosomal enzymes (Collins, 1999; Bertolini *et al*., 2001). The cysteinyl-containing leukotrienes ($LTC_4$, $LTD_4$ and $LTE_4$) cause intense vasoconstriction, bronchospasm, and increased vascular permeability. Generation of AA metabolites and their roles in inflammation are shown in Figure 2.

Other inflammatory mediators including vasoactive amines (histamine, serotonin), kinins, platelet-activating factor (PAF), nitric oxide (NO), and cytokines (Collins, 1999; Fantone and Ward, 1999, Steven and Lowe, 2000). Chemical mediators of inflammatory response are demonstrated in Table 2.

Histamine is the main pre-formed mediator of inflammation. It is released from mast cells, basophils and platelets, it causes transient dilatation of arterioles, increases permeability in venules, and is the primary cause of increased vascular permeability in the first hour after injury (Steven and Lowe, 2000).

Kinins play an important role in the inflammatory process, the production of kinins may also modulate migration of white blood cells and other cells into the inflammatory area (Regoli, 1987). Especially, bradykinin causes vasodilatation and increased vascular permeability. Its vasodilator action is partly a result of generation of $PGI_2$ and release of NO. It is a potent pain-producing agent, and its action is potentiated by the PGs (Rang *et al*., 2007).

PAF can elicit most of the features of inflammation, including enhances leukocyte adhesion to endothelium, increases vascular permeability, induces platelets aggregation and degranulation and stimulates synthesis of AA derivatives (Fishback, 2005).

NO probably has a net proinflammatory effect, it increases vascular permeability and PG production, and is a potent vasodilator (Rang *et al*., 2007).

Cytokines are polypeptide produced by many cell types, including mononuclear phagocytes, lymphocytes, endothelial and fibroblasts and chondrocytes. The production of cytokines by these cells is regulated by a number of stimuli, including other inflammatory mediators, and cell-cell contact. These products have additional effects that play important roles in initiating acute inflammation and maintaining chronic inflammatory response (Szekanecz and Koch, 2001; Michell and Cotran, 2003).

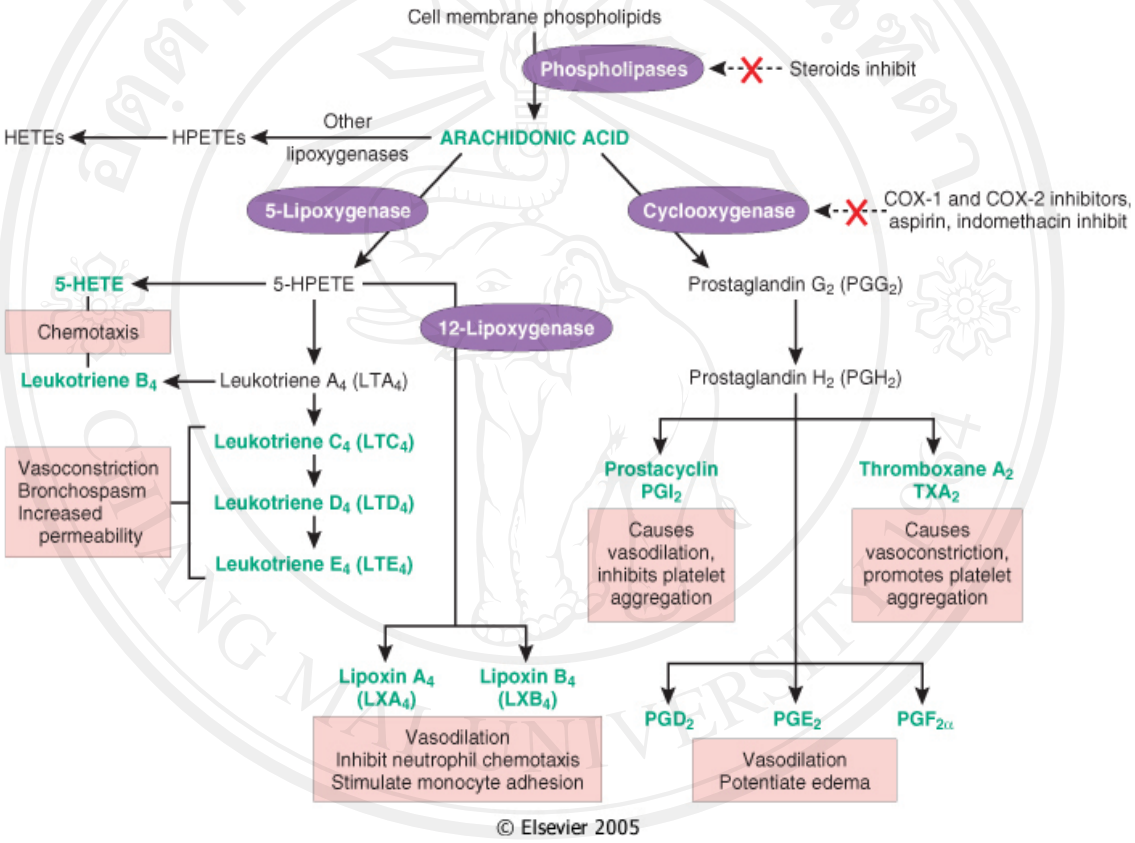


**Figure 2** Generation of arachidonic acid metabolites and their role in inflammation and the molecular targets of action of some anti-inflammatory drugs.

**Table 2** Chemical mediators of the inflammatory response

| Action | Mediators |
|---|---|
| Vasodilatation | PGs: $PGI_2$, $PGE_1$, $PGE_2$, $PGD_2$ |
| | NO |
| Increased vascular permeability | Histamine |
| | Complement components: C3a, C5a |
| | Bradykinin |
| | LTs, especially $LTC_4$, $LTD_4$, $LTE_4$ |
| | PAF |
| | Calcitonin gene-related peptide (CGRP) |
| | Substance P |
| Chemotaxis, leukocyte recruitment | C5a |
| and activation | $LTB_4$, lipoxins (LX): $LXA_4$, $LXB_4$ |
| | Bacterial products |
| Tissue damage | Neutrophil and macrophage lysosomal products |
| | Oxygen radicals |
| | NO |
| Pain | IL-1, IL-6, TNF-α |
| | $LTB_4$, $LXA_4$, $LXB_4$ |
| Fever | $PGE_2$, $PGI_2$ |
| | Bradykinin |
| | CGRP |

**Anti-inflammatory drugs**

The treatment of patients with inflammatory diseases involves two primary goals; first, the relief of pain which is often the presenting symptom and the major continuing complaint of the patients; and second, the slowing or in theory-arrest of tissue-damaging process (Furst and Robert, 2007). At present, anti-inflammatory drugs can be divided into aspirin and other NSAIDs and anti-inflammatory corticosteroids.

**1) NSAIDs**

All NSAIDs exhibit anti-inflammatory properties and are used as first-line agents for the symptomatic relief of inflammatory condition. The anti-inflammatory activity of NSAIDs is mediated chiefly through inhibition of biosynthesis of PGs by inhibition of COX-2 and/or COX-1 activities, and thereby inhibiting the synthesis of PGs and $TXA_2$. In general, NSAIDs provide only symptomatic relief from the pain and inflammation associated with disease and do not arrest the progression of pathological injury to tissue. They have been highly useful for treatment of acute, self-limited inflammatory conditions. The inhibition of COX-2 is thought to mediate, at least in part, the antipyretic, analgesic, and anti-inflammatory effects (Roberts II and Morrow, 2001). During therapy with these drugs, inflammation is reduced by decreasing the release of mediators from granulocytes, basophils and mast cells. The NSAIDs decrease the sensitivity of vessels to bradykinin and histamine, affect lymphokine production from T lymphocytes and reverse vasodilation (Furst and Hillson, 2001). Aspirin has long been used to reducing inflammatory response as well as reduces pain and fever (Gould, 2002). In addition, aspirin also interferes with the chemical mediators of the kallikrein system. It inhibits granulocyte adherence to damaged vasculature, stabilizes lysosomes, and inhibits the migration of polymorphonuclear leukocytes and macrophages into the site of inflammation (Furst and Robert, 2007). The side effects associated with NSAIDs are due to inhibition of the COX-1 enzyme leading to gastric ulcers, salt and water retention leading to edema by reducing the PG-induced inhibition of both the reabsorption of chloride and the action of antidiuretic hormone (Furst and Hillson, 2001; Roberts II and Morrow, 2001; Waller *et al*., 2005a). Therefore, agents that selective block COX-2 such as

meloxicam, celecoxib, and etoricoxib may offer a more favorable side effect profile yet still cause decrease in inflammation (Furst and Hillson, 2001; Gould, 2002). Anyhow, the cardiovascular side effects of those selective COX-2 inhibitors have been reported, leading to withdrawal couple of them from the market (rofecoxib, valdecoxib) (Solomon 2006; Hinz *et al*., 2007; Lange and Eberhart, 2007).

**2) Corticosteroids**

The action of corticosteroids historically was described as glucocorticoid (carbohydrate metabolism-regulating) and minerallocorticoid (electrolyte balance-regulating). The effects of corticosteroids such as prednisolone and dexamethasone are numerous, with widespread anti-inflammatory and immunosuppressive action. Most effects of corticosteroids are not immediate but become apparent after several hours because of the time required for changes in gene expression and protein synthesis (Schimmer and Parker, 2006). Glucocorticoids inhibit transcription of genes for synthesis of inducible COX-2, the inducible form of NO synthetase and of inflammatory cytokines such as IL-1, IL-2, and IL-5 and TNF-α and many others, mononuclear cell and neutrophil leukocyte migration into the inflammatory site (Waller *et al*., 2005c). In addition, the ability of these inflammatory cells to respond to and destroy phagocytosed microorganisms and release oxygen free radicals is also reduced. Corticosteroids stimulate production of the intracellular protein lipocortin-1 (or annexin-1). Lipocortin-1 inhibits phospholipase $A_2$ and therefore reduces the synthesis of PGs and LTs. It may also impair leukocyte migration in response to cytokine IL-1 (Stein and Pincus, 2001; Gould, 2002; Waller *et al*., 2005c). The pharmacological actions of corticosteroids in different tissues and many of their physiological effects seem to be mediated by the same receptor. Thus, the various corticosteriod derivatives used currently as pharmacological agents have side effects on physiological processes that parallel their therapeutic effectiveness (Schimmer and Parker, 2006). Adverse effects of long-term use and high dosages of corticosteroids including atrophy of lymphoid tissue and reduced numbers of white blood cells leading to an increased risk of infection and a decreased immune response, retarded growth in children, retention of sodium and water, often leading to edema (Gould, 2002).

***Phyllanthus emblica* Linn.**

*Phyllanthus emblica* Linn. (synonym: *Emblica officinalis* Gaertn.) is native to the tropics of South and Southeast Asia. It is also called Emblic, Emblic myrobalan, Indian Gooseberry, Malacca tree and Myrobalan. In Thailand, it is known as Ma-kham-pom (Figure 3). It is a deciduous tree of small or moderate size, up to 20 m high, with crooked trunk and spreading branches; bark greenish gray, peeling off in conchoidal flakes; branchlets glabrous or finely pubescent 10 to 20 cm long. Leaves imbricate when young, subsessile, 0.5 to 2.5 cm by 1.5 to 5.5 mm, closely set along the branchets, distichous, light green, glabrous, narrowly-linear, obtuse, having appearance of pinnate leaves; stipules minute, ovate, finely acute. Flowers small, monoecious, apetalous, greenish yellow, in axillary fascicles on the leaf-bearing branchets, often on the naked portion below the leaves, with fimbricate bracts at the base. Fruits sessile, 1.3 to 2.7 cm in diameter, flashy, globose or depress globose, with 6 longitudinal faint lines, glabrous, lucid, pale yellow; endocarp of triangular cocci, bony, dehiscent, with 3 short bundles of vascular tissue at the base, seeds 6 and trigonous (Department of Medical Sciences, Ministry of Public Health, 2000).

*P. emblica* is one of the most commonly used in many local traditional medicine systems including Ayurvedic medicine, Chinese, Indian as well as Thai herbal medicine. It has long been used to treat a broad spectrum of disorders including anorexia, indigestion, and anemia (Santisuk *et al*., 2005; Khan, 2009). The juice of the fresh bark mixed with honey and turmeric is given to treat gonorrhea. The leaf infusion with fenugreek seeds is given to treat chronic diarrhea. The pulp of the fruit is smeared on the head to dispel headache and dizziness (Perry, 1980). The fresh or dry fruit is used in traditional medicine for the treatment of diarrhea, jaundice and inflammatory disorder (Deokar, 1998; Khan 2009). In addition, the ripe fruits are astringent and extremely sour to taste. This plant is an essential ingredient of Ayurvedic preparation Triphala (*P. emblica* Linn., *Terminalia chebula* Retz., *Terminalia bellerica* Roxb.). This preparation has been described as an important health tonic for detoxification, rejuvenation, and balance, especially in summer season (Gaind *et al*., 1963). It is also a therapeutic agent for treatment of a variety of conditions such as headache, dyspepsia, constipation, liver conditions, fatigue, infections and assimilation, and is reported to possess many biological activities as

well, including antidiabetic (Sabu and Kuttan, 2002), antimutagenic (Kaur *et al*., 2002; Arora *et al*., 2003), antimicrobial (Mehta *et al*., 1993; Srikumar *et al*., 2007), radioprotective effect against gamma irradiation (Jagetia *et al*., 2002; Sandhya *et al*., 2006), immunomodulatory (Srikumar *et al*., 2005), anticancer (Kaur *et al*., 2005), antioxidant activities (Naik *et al*., 2005; Sandhya and Mishra, 2006), and chemoprevention (Deep *et al*., 2005), etc.

*P. emblica* is highly nutritious and is an important dietary source of vitamin C, minerals, and amino acids, such as calcium, phosphorus, iron, carotene, thiamine, riboflavin and niacin. The vitamin C is considered to be highly stable due to the presence of tannin and polyphenols (Morton, 1960). The seeds contain fixed oil, phosphatide and essential oil. The fruits, bark, and the leaves of this plant are rich in tannin. The root contains ellagic acid and lupeol and the bark contains leucodelphinidin (Thakur *et al.,* 1989). Chemical studies on this plant reveal several novel norbisabolane and bisabolane derivatives from the root (Zhang *et al*., 2000; Zhang *et al*., 2001b) as well as new galloyl esters of L-malic acid, mucic acid, and mucic acid 1,4-lactone from the fruit juice (Zhang *et al*., 2001c). Continuing investigation of the fruit juice results in the isolation of a new ellagitannin and phyllanemblinin A. In addition, five new ellagitannins (phyllanemblinins B-F) have been found in the leaves and branches (Zhang *et al*., 2001a).

The fresh juice of *P. emblica* fruit shows anti-atherosclerotic and hypolipidemic effects (Mathur *et al*., 1996). The acetone fruit extract possesses anti-mutagenic activity (Arora *et al*., 2003). Fifty percent alcoholic extract elicits hepatoprotective activity against country made liquor, paracetamol (Gulati *et al*., 1995), anti-tuberculosis drugs (rifampicin, isoniazid and pyrazinamide) (Tasduq *et al*., 2005a), carbon tetrachloride ($CCL_4$), thioacetamide (Tasduq *et al*., 2005b), and ethanol (Pramyothin *et al*., 2006). Moreover, this extract elevates the mitochondrial activity of human skin fibroblasts and promotes production of procollagen (Fujii *et al*., 2008). The butanol fraction of the water extract of *P. emblica* fruits has been found to produce cytoprotective action on gastric ulcer formation (Bandyopadhyay *et al*., 2000). The ethanol fruit extract shows antimicrobial (Ahmad *et al*., 1998), gastroprotective (Al-Rehaily *et al*., 2002), antiproliferative (Khan *et al*., 2002), antioxidant, immunomodulator (Sai Ram *et al*., 2002), analgesic, and antipyretic

activities (Perianayagam *et al*., 2004). Furthermore, the ethyl acetate fruit extract provokes hypocholesterolemic (Kim *et al*., 2005) and NO scavenging activities (Kumaran and Karunakaran, 2006). The methanol extract shows anti-ulcerogenic (Sairam *et al*., 2002), hepatoprotective activity against $CCl_4$ (Lee *et al*., 2006), and chemopreventive potential for hepatocarcinogenesis (Sultana *et al*., 2008). The fruit extract (ethyl acetate:methanol fraction) shows antinociceptive property in high fat diet-fed/low dose streptozotocin-induced diabetic neuropathy in rats (Kumar *et al*., 2009). In addition, the various extracts from the fruit shows anti-carcinogenesis (Jeena *et al*., 1999), hepatoprotective activity against $CCl_4$ (Jose and Kuttan, 2000), anti-tumor (Jose *et al*., 2001), immunomodulator in adjuvant-induced arthritis rat model (Ganju *et al*., 2003), radioprotective effect against gamma irradiation (Singh *et al*., 2005, 2006), hypotensive (Ishag *et al*., 2005), as well as chondroprotective potential (Sumantran *et al*., 2008).

Experiments conducted with the water fraction of methanol leave extract of *P. emblica* revealed its anti-inflammatory activity on carrageenan- and dextran-induced rat hind paw edema (Asmawi *et al*., 1993). Moreover, the methanol, tetrahydrofuran, 1,4-dioxane extract and the water fraction of methanol leave extract have been found to have inhibitory activity on the migration of human polymorphonuclear leukocytes (PMNs) (Asmawi *et al*., 1993; Ihantola-Vormisto *et al*., 1997), and the diethyl ether leave extract has been shown to possess inhibitory activity on platelets by inhibiting the $TXB_2$ production (stable product of $TXA_2$) in platelets during blood clotting and thereby inhibiting platelet aggregation (Ihantola-Vormisto *et al*., 1997). In addition, the toxicity study of *P. emblica* has been reported. Single intraperitoneal dose of water leave extract shows 50% lethal dose ($LD_{50}$) of 0.415 and 0.288 g/kg body weight of male and female mice, respectively. Subacute toxicity test in both male and female mice of water leave extract at the dose of 0.1 and 0.5 g/kg body weight given orally for 10 weeks does not produced toxicity and the mice have normal histopathology of internal organs (Itthipanichpong *et al*., 1987). However, the scientific data to support the reputed anti-inflammatory activity and toxicity of the standardized water extract from the fruits prepared according to the Thai Herbal Pharmacopoeia (THP) have not yet been reported.


**Figure 3** *Phyllanthus emblica* Linn. (Family Euphorbiaceae), Ma-kham-pom.

### 1.3 Hypothesis

The hypothesis of this study is that the standardized water extract of the fruits of *P. emblica* prepared according to the Thai Herbal Pharmacopoeia possesses anti-inflammatory and related activities (i.e. analgesic and antipyretic) as well as chondroprotective and anti-ulcerogenic effects. In addition, this standardized water extract is nontoxic.

### 1.4 Purpose of the study

The purposes of the present study were to investigate the following activities of *P. emblica*:

1. Anti-inflammatory, analgesic and antipyretic activities
2. Inhibitory effect on cyclooxygenase enzyme
3. Chondroprotective activity
4. Anti-ulcerogenic activity
5. Acute and chronic oral toxicity

### 1.5 Research design

**Inflammatory models**

**1. Ethyl phenylpropiolate (EPP) and arachidonic acid (AA)-induced ear edema in rats**

Edema is a useful parameter to look at when testing for agents which may be active in treating acute inflammation (Sedgwick and Willoughby, 1989). Rat ear edema induced by EPP is suggested to serve as a more useful model for rapid *in vivo* screening of anti-inflammatory activity. By using EPP, the mechanism involved can be suggested. EPP causes release of many inflammatory mediators such as kinin, serotonin and PGs (Brattsand *et al*., 1982). AA-induced ear inflammation in mice has been reported to be sensitive in detecting the anti-inflammatory action of lipoxygenase inhibitors (Young *et al*., 1984; Carlson *et al*., 1985). Lipoxygenase metabolites, especially LTs, have an important role in producing vascular permeability and edema formation whereas COX products have low or no activity (Di Martino *et al*., 1987).

**2. Carrageenan-induced hind paw edema in rats**

Carrageenan-induced inflammation is useful to detect orally active anti-inflammatory agents (Di Rosa *et al*., 1971). Carrageenan is a sulphate polysaccharide which has been fractionated with potassium chloride into two separate components, kappa and lambda carrageenan (Di Rosa, 1972). The lambda carrageenan is more active in eliciting either acute or chronic inflammatory responses.

The advantage of carrageenan-induced edema in comparison with the edema elicited by other phlogistic agents is its responsiveness to doses of all clinical used anti-inflammatory drugs at well below the toxic level, with the degree of edema inhibition being in a dose-related manner (Winter *et al*., 1962). The edema formation induced by carrageenan is mediated by the initial release of histamine and serotonin followed by the release of bradykinin during the 1-2 h after carrageenan injection (Crunkhorn and Meacock, 1971). The second phase of inflammation is due to the release of PGs which occurs 2-2.5 h after carrageenan injection and lasts about 6 h (Winter *et al*., 1962; Vinegar *et al*., 1969). It is important when using this model to assess the effect of the potential anti-inflammatory agent at the appropriate time during the swelling of the hind paw. This test is excellent for detecting inhibitors of COX (Sedgwick and Willoughby, 1989).

**3. Cotton pellet-induced granuloma formation in rats**

The inflammatory granuloma is a typical feature of established chronic inflammatory reaction (Spector, 1969). The response to a subcutaneously implanted cotton pellet in rat has been divided into at least three phases. These consist of (1) a transudative phase, defined as the increase of vascular permeability and thereby in wet weight of the pellet which has occurred during the first three hours after implantation, (2) an exudative phase, defined as a leakage of fluid and proteins from the bloodstream around the granuloma and occurring between 3 and 72 hours after implanting the pellet, and (3) a proliferative phase, measured as the increase in dry weight of the granuloma which occurs between three and six days after implantation (Swingle and Shideman, 1972). This method is generally used to evaluate the interfering capacity of agents on the proliferative phases of inflammatory process.

Anti-inflammatory drugs can reduce transudative weight probably via their ability to inhibit the permeability response of the blood vessels around the cotton pellet implantation. They can also effectively inhibit granuloma formation probably due to interference with proliferative component of inflammatory process. Moreover, the NSAIDs, such as aspirin, elicit only a slight inhibition whereas steroidal anti-inflammatory drugs have a strong inhibition on both transudative and proliferative phase of inflammation (Swingle and Shideman, 1972). Steroids, e.g. prednisolone, can prevent or suppress inflammatory reactions. Steroids can stimulate protein synthesis in the liver and also influence peripheral catabolism of lymphoid and connective tissues, muscle, fat and skin (Schimmer and Parker, 2006). The loss of body weight gain and thymus weight of rats in long-term prednisolone treatment may be due to increased protein catabolism and lymphoid tissue destruction.

The phagocytosis process is believed to contribute to tissue damage by releasing large number of substances include lysosomal enzymes (e.g., phospholipase enzymes) and oxygen radicals. The ability of macrophages to defend tissue homeostasis and participate in inflammatory responses depends on their ability to mobilize granule-membrane proteins and granule content into their external milieu and into phagosomes by regulated secretory processes (Tapper, 1996). Thus, serum alkaline phosphatase (ALP) activity can be assessed in this model. ALP is a lysosomal enzyme widely distributed in many tissues, including the osteoblasts (the bone-building cells), the cell lining the sinusoids and bile canaliculi in the liver. The activity of lysosomal enzymes is markedly increased during inflammation (Nishikaze *et al*., 1980). The role of lysosomal enzymes as mediators of inflammation is well documented (Hsueh, 1979). ALP activity in pouch wall is elevated during cotton pellet granuloma formation on the seventh day and decreased on the fourteenth day, when healing occurs (Nishikaze *et al*., 1980). Measurement of serum ALP activity of rats implanted with cotton will indicate the activity of agents on the production and release of ALP in chronic inflammation. Corticosteroids stabilize lysosomes, thereby reducing the local release of proteolytic enzymes and hyaluronidase, other substances that contribute to tissue swelling (Berne and Levy, 2004).

**Algesic models**

**1. Formalin test in mice**

The formalin test is a valid and reliable model of nociception. This test was introduced by Dubuisson and Dennis (1977) and the subcutaneous injection of formalin produces a biphasic pain response. On the basis of the response pattern, two distinct periods of intensive licking activity are an early (0-5 min after injection) and a late response (20-30 min after injection) (Dubuisson and Dennis, 1977; Hunskaar *et al*., 1985; Hunskaar and Hole, 1987). The response of early phase is believed to represent a direct chemical stimulation of nociceptors (Dubuisson and Dennis, 1977; Hunskaar *et al*., 1985; Tjolsen *et al*., 1992), due to the irritant effect of formalin on sensory C fibers (Heapy *et al*., 1987; Tjolsen *et al*., 1992). The late phase response is most likely secondary to the development of an inflammatory response and the release of algesic mediators (Hunskaar and Hole, 1987). Experimental results have indicated that substance P and bradykinin participate in the early phase, while histamine, serotonin, PGs, and bradykinin are involved in the late phase (Shibata *et al*., 1989).

**Pyretic model**

**1. Yeast-induced hyperthermia in rats**

The pyrexia, induced in rat by subcutaneous injection of brewer's yeast, has been used to determine antipyretic activity of test substances (Teotino *et al.,* 1963). The pyrexia reaches its peak at 18 h after induction and assessment is also made at this period. It has been postulated that many chemical neuromediators are involved in hypothalamic regulation of body temperature. $PGE_2$ is one of the most potent pyretic agents known and elevated concentration of $PGE_2$ has been found in cerebrospinal fluids taken from pyretic patients or animals (Davies *et al*., 1984).

**Chondroprotective model using cartilage explant culture**

Articular cartilage composes of chondrocytes, extracellular matrix (ECM) and water. Chondrocytes are important in the control of matrix turnover through production of collagen, proteoglycans and enzymes for cartilage metabolism. Water is the major component of cartilage, contributing up to 85% of the wet mass. Extracellular matrix, has characterized many skeletal macromolecules including classes of collagens, proteoglycans and glycoproteins.

Proteoglycans have a core protein and at least one covalently attached glycosaminoglycans (GAGs). GAGs are the polysaccharides which contain acetylated amino sugars (*N*-acetylgalactosamine (GalNAc) or *N*-acetylglucosamine (GlcNAc)) and a uronic acid (glucuronic acid or iduronic acid). Chondroitin sulfate is the most common glycosaminoglycan chains. Chondroitin sulfate is a disaccharide structure containing GalNAc (amino sugar) links with the glucuronic acid (a uronic acid). Other chains include dermatan sulfate, keratan sulfate, heparan sulfate and heparin.

One glycosaminoglycan, hyaluronan (HA), is synthesized at the cell surface and is not attached to a core protein. The biological functions of HA include maintenance of the elastoviscosity of liquid connective tissues such as joint synovial and eye vitreous fluid, control of tissue hydration and water transport, and supramolecular assembly of proteoglycans in the ECM (Necas *et al*., 2008). Proteoglycan loss is a rapid event following proinflammatory stimulation but it can be readily replaced once the stimulus is removed (Rowan, 2001).

Collagen is the major fibrillar protein of ECM. This protein is important because it is responsible for tissue strength and resilience. Defects in the synthesis and degradation of collagen contribute to a number of diseases including arthritis, osteoporosis and fibrotic diseases (Rowan, 2001).

Several studies have reported that IL-1β and TNF-α are the key proinflammatory cytokines mediating cartilage degradation in patients with RA and OA. IL-1β and TNF-α participate in these processes by stimulating chondrocytes and synoviocytes to produce matrix proteases, chemokines, nitric oxide and eicosanoids such as PGs and LTs. Stimulation of cartilage explants with IL-1β has been shown to cause wide spread matrix degradation, including loss of tissue proteoglycans and

collagens. These events result in a reduced amount of GAGs in the matrix, decreased binding between GAGs and collagen II, and an increase in the amount of water in the matrix (Gowen *et al*., 1984; Brennan *et al*., 1992; Feldmann *et al*., 1996; Martel-Pelletier, 1998; Pelletier *et al*., 2001; Csaki *et al*., 2009).

**Ulcerogenic models**

Anti-ulcerogenic activity is generally assessed by testing the effect of test substances on rats with gastric ulcerations induced by ethanol/hydrochloric acid (Mizui and Doteuchi, 1983), indomethacin (Nwafor *et al*., 2000) or restraint water immersion stress (Takagi and Okabe, 1968).

**1. Ethanol/hydrochloric acid (EtOH/HCl)-induced gastric lesions**

The gastric lesions are hemorrhagic as well as the necrotic aspects of the tissue injury, caused by the direct action of ethanol (Oates and Hakkinen, 1988). The factors involved in the formation of ethanol-induced gastric lesions may be multifactorial, by impairment of defensive factors such as mucus secretion (Kuwata *et al*., 1985), and bicarbonate secretion (Flemstrom, 1987; Crampton, 1988), and of mucosal circulation (Trier *et al*., 1987). It has been reported that ethanol caused gastric lesions by decreasing gastric mucosal blood flow and gastric acid has little part in such lesion formation (Szabo, 1987). In addition to superficial aggressive cellular necrosis, ethanol can cause release of tissue-derived mediators such as histamine and $LTC_4$ (Oates and Hakkinen, 1988). These mediators act on gastric microvasculature, triggering a series of events that result in mucosal and possibly submucosal tissue destruction. It has been reported that LT antagonists and 5-LOX inhibitors are capable of inhibiting ethanol-induced gastric ulceration in rats (Parnaham and Brune, 1987). The EtOH/HCl ulcer model is commonly employed for determining whether the anti-gastric ulcer activity involves the effects on gastric mucosal protective factors, and the substance with cytoprotective activity will be able to prevent gastric lesions in this model. Acid anti-secretory agents such as histamine 2 ($H_2$)-receptor antagonists (e.g. cimetidine, ranitidine), can prevent the gastric lesions induced by EtOH/HCl, and it is suggested that the activity is attributable to cytoprotective activity and partly to their ability to suppress acid secretion (Miyata *et al*., 1991).

**2. Indomethacin-induced gastric lesions**

Non-steroidal anti-inflammatory drugs like indomethacin are known to induce gastric lesions via several mechanisms. NSAIDs disrupt the normal gastric mucosal barrier of bicarbonate and hydrophobic mucus by disturbance of PG synthesis resulting from inhibition of constitutive COX isoenzyme, COX-1 (Vane, 1971; Selling et al., 1987; Hayllar and Bjarnoson, 1995; Hawkins and Hanks, 2000). In stomach, PGs have a vital protective role, maintaining mucosal blood flow, stimulating secretion of bicarbonate and mucus and regulating mucosal cell turnover and repair (Hayllar and Bjarnoson, 1995). High concentration of PGs, especially $PGE_2$ and $PEI_2$, are present in the normal gastric and duodenal mucosa and they are responsible for mucus production and inhibition of gastric acid secretion (Robert *et al*., 1983; Valle, 2005). This principle pathway of inhibition of biosynthesis of cytoprotective PGs e.g., $PGE_2$ and $PGI_2$ results in over production of LTs and other products of 5-LOX pathway (Rainsford, 1987). LTC4 could mediate gastric mucosal damage both its vasoconstrictive actions and its effects on vascular permeability promoting vascular stasis and subsequent reduction in tissue perfusion (Whittle *et al*., 1985; Pihan *et al*., 1988). Some leukotriene antagonists and 5-lipoxygenase inhibitors are capable to protect the gastric mucosa against lesions induced by oral or parenteral administration of NSAIDs (Rainsford, 1987). In addition, it has been reported that the erosions induced by indomethacin are prevented by acid anti-secretory agent such as $H_2$-receptor antagonists (Kuratani *et al*., 1992).

**3. Restraint water immersion stress-induced gastric lesions**

Water immersion and hypothermic restraint stress are widely used as experimental models to induce acute stress ulcers in rats (Takagi *et al.*, 1963; Senay and Levine, 1967). Gastric ulcers can be induced in experimental animals and humans by physical or psychological stress (Takagi and Okabe, 1968). The pathogenesis of stress-induced gastric lesions is poorly understood and it may involve vascular, endocrine, mucosal and neurogenic factors (Breckenridge *et al*., 1959; Feldman and Sabovich, 1980). The main factors in the gastric lesion formation include: increase of acid secretion (Brodie *et al*., 1962; Menguy, 1969; Kitagawa

*et al*., 1979) and increase of gastric motility (Watanabe, 1966; Yano *et al*., 1978; Garrick *et al*., 1986), decrease of gastric mucosal blood flow (Guth and Kozbur, 1968; Guth, 1972; Hase and Moss, 1973; Kitagawa *et al*., 1979) and decrease of alkaline secretion (Takeuchi *et al*., 1990).

**Toxicity test**

Toxicity testing is intended to provide information of the safety of a test substance before using it in clinical practice. For this, the species chosen for testing should be most similar to the human in the way it handles the test article pharmacodynamically. Substaintial differences in absorption, distribution, metabolism, or elimination between test species and the target species (e.g. human) will reduce the predictive value of the test results. From a practical standpoint, often the pharmacokinetics are unknown in humans or a variety of available test species at the time of species selection. For this reason, testing is usually conducted in at least two species. Generally, one of those species is usually a rodent and the other is a nonrodent. The two most commonly used rodent species are mice and rats, and often toxicity testing is conducted in both of those species. The period of administration of the test substance to animals will depend on the expected period of clinical use as shown in Table 3 (WHO, 2000).

**Table 3** Commonly used ranges of administration periods

| Expected period of clinical use | Administration period for the toxicity study |
|---|---|
| Single administration or repeated administration for less than one week | 2 weeks to 1 month |
| Repeated administration, between one week to four weeks | 4 weeks to 3 months |
| Repeated administration, between one to six months | 3 to 6 months |
| Long-term repeated administration for more than six months | 9 to 12 months |

**1. Acute toxicity**

The procedure has been recommended by the World Health Organization (WHO) guideline (WHO, 2000) and the Organization of Economic Co-operation and Development (OECD) guideline for testing of chemicals (OECD, 2001). In the assessment and evaluation of the toxic characteristics of a substance, determination of acute oral toxicity is usually an initial step. It provides information on health hazards likely to arise from a short term exposure by the oral route. Data from an acute study may serve as a basis for classification and labeling. It is an initial step in establishing a dosage regimen in subchronic and other studies and may provide initial information on the mode of toxic action of a substance. Acute oral toxicity is the adverse effect occurring within a short time of oral administration of a single dose of a substance or multiple doses given within 24 hours.



**2. Chronic toxicity**

The procedure has been suggested by the WHO guideline (WHO, 2000) and OECD guideline for testing of chemicals (OECD, 1981). The objective of a chronic toxicity study is to characterize the profile of a substance in a mammalian species following prolonged and repeated exposure over a portion of the average life span of

the experimental animals. Chronic study will provide information on target organs and physiological function, the possibilities of cumulation, and can provide an estimation of no effect level of exposure which can be used in selecting dose levels for chronic studies and for establishing safety criteria for human exposure. For example, repeated administration between one to six months of clinical use, thus six to nine months is the administration period for the toxicity study (WHO, 2000). The toxic effects of the test substance basically can be defined by physical examination, daily observations, ophthalmological examination, determination of food and water consumption, body and organ weight, hematology, biochemical and organ function tests, and pathological studies. Where possible, these parameters should be evaluated prior to initiation of the study to obtain the baseline information on the animals.