CHAPTER 1

Introduction

1.1 Statement and significance of the problem

Arthritis is an inflammation of a joint afflicting many patients worldwide. There are over 100 different forms of arthritis, such as gout (a crystal-induced arthritis and an acute arthritis), osteoarthritis (a cartilage disorder), rheumatoid arthritis (a systemic disorder with arthritic manifestations), juvenile rheumatoid arthritis (JRA), infectious arthritis, lupus, fibromyalgia, etc. Among these, osteoarthritis (OA) and rheumatoid arthritis (RA) are two of the most common arthritis (Watson *et al*., 2003; De Filippis *et al*., 2004; Ahmed *et al*., 2005).

OA is a degenerative joint disease but RA is an autoimmune inflammatory disease. The degree of inflammation, destruction of the joint and the mortality rate in RA is much greater than in OA (Watson *et al*., 2003). It has been reported that RA patients have a higher risk of death and vascular events when compared with OA patients or no arthritis diagnostic people (Watson *et al*., 2003). Many studies have consistently demonstrated that RA can cause an increase mortality from cardiovascular disease and pulmonary fibrosis (Wolfe *et al*., 2003; Maradit-Kremers *et al*., 2005a; Maradit-Kremers *et al*., 2005b; Young *et al*., 2007 Bergstrom *et al.*, 2009).

The goals of RA treatments are to reduce pain and inflammation, prevent or control joint damage and prevent loss of function. The current drug therapies for RA are non-biologic disease-modifying anti-rheumatic drugs (DMARDs), biologic DMARDs (TNF-α inhibitors and IL-1 receptor antagonists), glucocorticoids and non-steroidal anti-inflammatory drugs (NSAIDs) (Lee and Kavanaugh, 2003). However, some RA patients do not respond to these drugs. The adverse effects from these drugs are still the major problem of RA treatment. Biologic DMARDs, non-biologic DMARDs and glucocorticoids, cause serious adverse effects to user by suppressing immune system

leading to increase the risk of infection. NSAIDs are widely used in the first stage of RA with mild symptom for relieving pain and reducing inflammation in the RA joints. However, prolonged use of NSAIDs may increase gastrointestinal complications such as gastric ulcer and bleeding. Therefore, it is interesting to investigate a herbal medicine with claimed anti-arthritic property by verifying beneficial effects on joint inflammation and other symptoms of arthritis such as pain. As most plants have no irritating effects on gastric mucosa, gastro-protective effect of the herbal medicine should be examined as well.

1.2 Rheumatoid arthritis (RA)

RA is a chronic systemic inflammatory disease that presents as a symmetric polyarthritis associated with swelling and pain in multiple joints or the membrane around the joint. Joints of the hands and feet are the most often attacked in RA. The chronic inflammation in RA causes synovial cell hyperplasia that finally leads to cartilage and bone destruction. The prevalence of RA is about 1% and is more common in women than men (Gabriel, 2001). RA can start at any age but most commonly at the age between 20-45 years. The cause of RA is unknown. However, the factors such as genetic susceptibility, sex and age, smoking, infectious agents, hormonal, dietary, socioeconomic, and ethnic factors are considered the main risk factors for the disease (Alamanos *et al.*, 2005). Most of these factors are likely to be associated with both severity and occurrence of disease (Alamanos *et al.*, 2005).

The possible mechanisms to initiate inflammatory and bone destruction in RA are shown in Figure 1.1. The most important cells in RA synovium are synovial macrophages, synovial fibroblasts, and infiltrating T lymphocytes. Another important cell populations include B lymphocytes, dendritic cells, plasma cells, mast cells, and osteoclasts (Tran *et al*., 2005). Komatsu and Takayanagi (2012) separated the arthritis progression into three phases of the initiation phase, inflammatory phase and bone destructive phase.

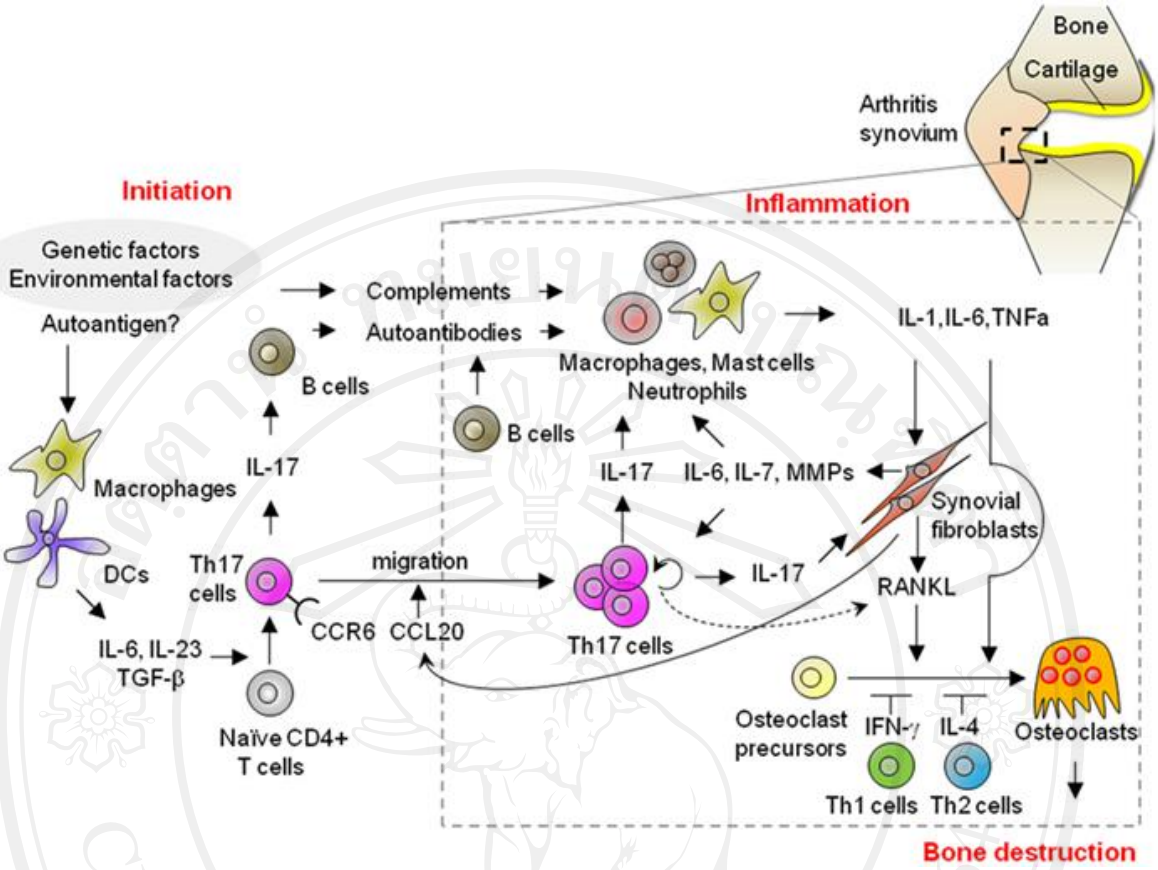

Figure 1.1 Possible mechanisms of the initiation, inflammatory, and bone destruction phases in RA (Komatsu and Takayanagi, 2012).

The initiation phase is triggered by an autoimmune response to risk factors or autoantigen. In this phase, macrophages are the initiators of the pathogenic cascade of RA. The stimulation of macrophages by many risk factors in the synovial tissue leads to the releasing of proinflammatory cytokines involved in inflammation. Cytokines produced by macrophages and dendritic cells can stimulate $CD4^+$ T lymphocytes to Th17 cells. Th17 cells release IL-17 that can activate B cells to produce autoantibodies and complements (Kotake *et al*., 1999; Koenders *et al*., 2006).

The inflammatory phase starts when the signs of inflammation such as swelling are found in joints and continues until any structural changes occur (Komatsu and Takayanagi, 2012). Synovial fibroblasts contribute to Th17 immunity in the inflammatory phase of arthritis by promoting the migration of Th17 cells into the inflammatory joint, and then homeostatic proliferation and concomitant increase in IL-17 production (Komatsu and Takayanagi, 2012). IL-17 can activate the inflammatory cell such as synovial macrophages, mast cells, and neutrophils to produce IL-1, IL-6, and TNF-α. These cytokines stimulate synovial fibroblast to produce IL-6, IL-7, and matrix metalloproteinases (MMPs) that in turn affect the inflammatory cells (Bingham, 2002; Komatsu and Takayanagi, 2012). However, IL-17 also directly activate synovial fibroblasts to produce IL-6, IL-7, and MMPs that affect other inflammatory cells (Kinne *et al*., 2000). The inflammation in RA can occur in tissues around the joints, such as tendons, ligaments, and muscles.

The final phase is the bone destruction phase that occurs when bone and cartilage are damaged. $CD4^+$ T-cells also play a key role in the pathogenesis of RA, not only the initiation and inflammation phase but also in bone destruction phase. Activated T cells release IL-17 that directly activates synovial macrophages and B cells resulting in the releasing of IL-1, IL-6, and TNF-α in the inflamed synovium (Komatsu and Takayanagi, 2012). These cytokines cause the recruitment of osteoclast precursors to inflamed joints and stimulate the differentiation of osteoclasts (Brennan *et al*., 2008; Komatsu and Takayanagi, 2012). Osteoclasts involve in bone remodeling and mediate bone loss in pathologic conditions by increasing their resorptive activity. The cytokines TNF-α, IL-1, and IL-6 stimulate the expression of receptor activator of nuclear factor-κB ligand (RANKL) on synovial fibroblasts and T cells. It has been reported that

RANKL is produced by synovial B cells in RA (Takayanagi, 2005). Th17 cells also express RANKL on their membranes, which partly contribute to the enhance osteoclastogenesis (Corrado *et al*., 2013). Binding of RANKL to the RANK receptor on osteoclast precursors and mature osteoclasts, leads to stimulation of several signaling pathways of osteoclast differentiation and activation (Figure 1.2).

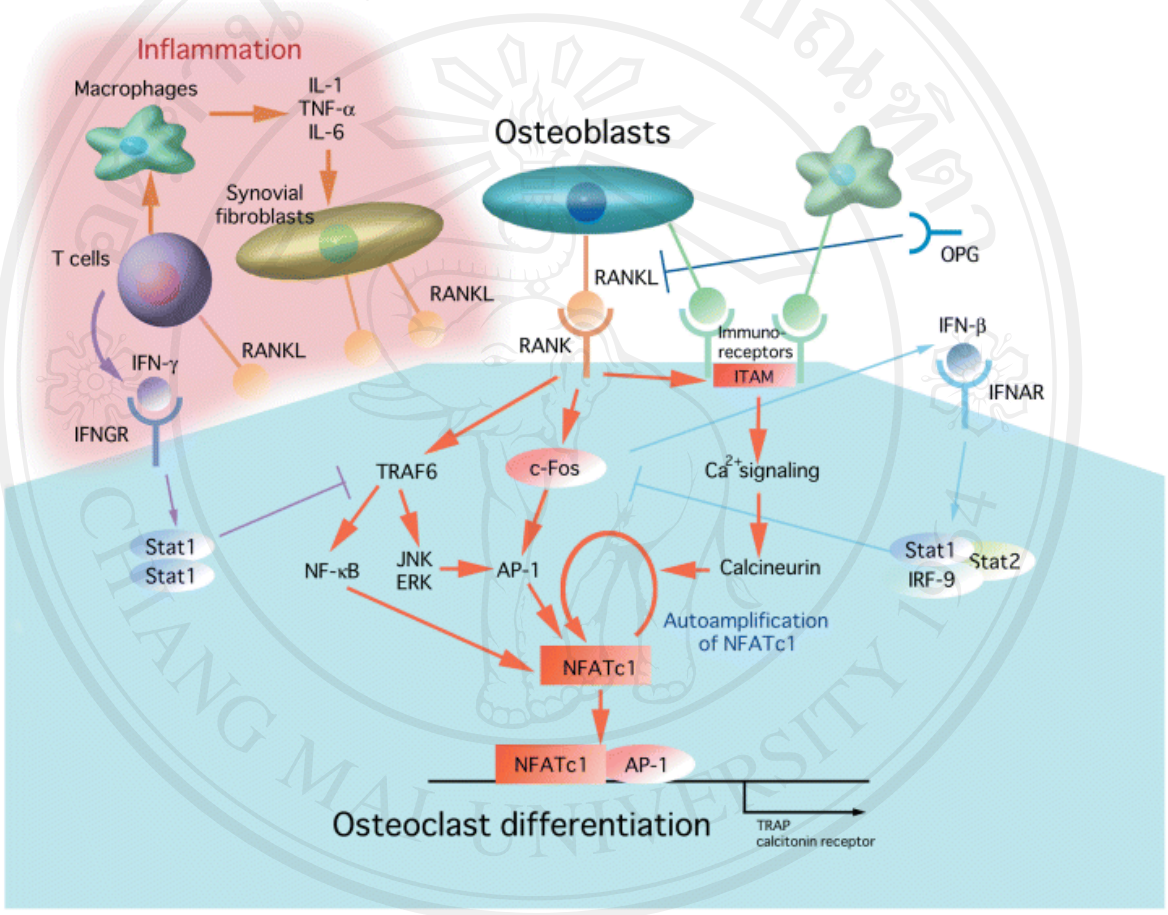

Figure 1.2 Signaling crosstalk between immune and skeletal systems in osteoimmunology (Takayanagi, 2005).

1.3 Inflammatory mediators in RA

TNF-α is proinflammatory cytokine produced by activated macrophages and fibroblasts in the synovial membrane. TNF-α is a key molecule in the control of the inflammatory changes that occur in the RA synovium (Matsuno *et al*., 2002). The roles of TNF-α on the pathogenesis of RA include stimulation of the release of chemokines that attract leukocytes from the blood into the inflamed tissue. It also up regulates adhesion molecules, induces other proinflammatory cytokines production (IL-1 and IL-6), and angiogenesis. The production of proteolytic and MMPs enzymes that involved in cartilage and subchondral bone destruction as well as to stimulate prostaglandin (PG) E_2 (PGE_2) production are the role of TNF-α in RA (Brennan *et al*., 2008).

IL-1 consists of three members of IL-1β, IL-1α, and IL-1 receptor antagonist (IL-1Ra). Under physiological conditions, the activities of IL-1β and IL-1α are balanced by IL-1Ra (Joosten *et al*., 1999). IL-1β is secreted and acts on other cells to produce its biological actions. It induces cellular responses by binding to IL-1 receptor type I (IL-1RI), whereas IL-1Ra inhibits the effect of IL-1β (Joosten *et al*., 1999). IL-1 mediates the osteoclastogenic effect of TNF-α by enhancing stromal cell expression of RANKL and directly stimulating differentiation of osteoclast precursors (Wei *et al*., 2005). Joosten and colleagues reported that IL-1β involves in joint destruction in mice induced arthritis by collagen and that treatment with anti-IL-1 significantly decrease cartilage and joint destruction in this model (Joosten *et al*., 1999).

IL-6 is a cytokine produced by various cells type including T and B cells, monocytes, fibroblasts, osteoblasts, osteoclasts, keratinocytes, endothelial cells, mesengial cells and some tumor cells (Kishimoto, 1989). It can facilitate autoimmune phenomena, amplify acute inflammation, and promote the evolution into a chronic inflammatory state including bone resorption in RA (Fonseca *et al*., 2009; Mori *et al*., 2011). IL-6 can activate cells through both membrane-bound (IL-6R) and soluble receptors (sIL-6R) (Dayer *et al.*, 2010). It has been reported that the elevation of IL-6 levels and sIL-6R in the synovial fibroblast (SF) of RA patients can increase the risk of joint destruction. IL-6 binding with it receptor, sIL-6R, can stimulate pannus development through increased vascular endothelial growth factor (VEGF) expression and increased bone resorption as

a result of osteoclastogenesis (Dayer *et al.*, 2010). Recently, Mori and colleagues proposed that IL-6–STAT3 pathway involves in the promotion of inflammatory cytokines amplification and joint destruction. Major proinflammatory cytokines including IL-6 directly stimulate STAT3 that causes further RANKL expression and promotes osteoclastogenesis and joint destruction (Mori *et al*., 2011).

The cytokines, TNF-α, IL-1β, and IL-6 involve in joint destruction and bone resorption in RA. Many studies reported that, these cytokines stimulate osteoclasts differentiation in a synergistic fashion and induce the expression of RANKL in osteoclasts leading to bone loss (Zhang *et al*., 2001; Ragab *et al*., 2002; Kwan Tat *et al*., 2004; Wei *et al*., 2005). TNF-α, IL-1, and IL-6 blockade are not only able to prevent the structural joint damage, but also to prevent bone loss in RA (Corrado *et al*., 2013).

IL-17 has been implicated in the pathogenesis of inflammatory bone and joint damage through induction of MMPs and osteoclasts, as well as inhibition of proteoglycan synthesis (Stamp *et al*., 2004). The binding of IL-17 to its receptors expressed on epithelial, endothelial, and fibroblastic stromal cells triggers the activation of transcription factors, which in turn results in the secretion of various proinflammatory cytokines (Fossiez *et al*., 1996; Paradowska *et al*., 2007).

Other cytokines such as IL-8**,** granulocyte-monocyte colony-stimulating factor (GM-CSF), and IL-15 also increases in RA. IL-8 involves in cellular recruitment, GM-CSF involves in macrophage development, IL-15 involves in T cell proliferation. IL-23 involves in increasing Th17 cell differentiation (Aggarwal *et al*., 2003; Tesmer *et al*., 2008)

Multiple studies have shown that the expression of several cytokine genes associate with nuclear factor-κB (NF-κB). NF-κB activation is activated in the RA synovium and controls transcription of inflammatory genes associated with inflammation, including TNF-α, IL-1, IL-6, IL-8, cyclooxygenase-2 (COX-2), inducible nitric oxide sythetase (iNOS) and intercellular adhesion molecule-1 (ICAM-1) (Han *et al*., 1998).

The expression of COX-2 and iNOS and the production of PGs and nitric oxide (NO) are regulated by many cytokines. PGs are involved in pain sensitization and localized

inflammation. PGE_2 produces from COX-2 by synoviocytes also participates in destructive mechanisms in rheumatoid joint (Sano *et al*., 1992; Bingham, 2002). It has been reported that PGE_2 plays major roles in angiogenesis through the expression of VEGF in the rheumatoid synovium (Ben-Av *et al*., 1995). PGE_2 participates in osteoclastogenesis through the expression of RANKL in RA synoviocytes and infiltration of T cells (Kotake *et al*., 1999).

NO plays an important role in the cascade of inflammatory process, including RA. It has been reported that the concentrations of nitrite in serum and synovial fluids of the rheumatoid and osteoarthritic patients are significantly higher than in the control group (Farrell *et al*., 1992). Previous evidence suggests that NO contributes to T cell dysfunction in autoimmune diseases (Nagy *et al*., 2010). NO also mediates many different cell functions at the site of synovial inflammation, including cytokine production, signal transduction, mitochondrial functions and apoptosis. NO derived from macrophages may enhance bone loss by augmenting the cytokine-induced MMP-1 production in osteoblasts and subsequent bone destruction (Lin *et al*., 2003). It has been suggested that iNOS pathway is essential for IL-1-induced bone resorption and the effect of NO may be mediated by modulating IL-1-induced nuclear activation of NF-κB in osteoclast precursors (van't Hof *et al*., 2000).

1.4 Pharmacotherapy for rheumatoid arthritis

The goals in managing RA are to prevent or control joint damage, prevent the loss of function, and reduce pain. The summary of drugs used for RA treatment is shown in Table 1.1 that including NSAIDs, glucocorticoids, DMARDs, and biologic DMARDs (Lee *et al.*, 2003). The immunosuppressive drugs such as leflunomide is used to relieve disease process in some patients that do not respond to other drugs (Herrmann *et al*., 2000).

NSAIDs are the drugs widely used to control pain and inflammation in RA. The major problem with traditional NSAIDs is the gastrointestinal (GI) and renal (Jones, 2001) side effects. The side effects of NSAIDs are various gastrotoxicity, ranging from clinically unimportant disturbances to serious complications such as erosions, peptic ulcer, and bleeding (Del Favero *et al*., 1991). In patients with RA, the treatment with a selective COX-2 inhibitor reduces upper GI events when compared with naproxen, a nonselective NSAID (Bombardier *et al*., 2000). However, selective COX-2 inhibitors may lead to an increase in thrombotic cardiovascular events (Fries *et al.*, 2005; Grosser *et al.*, 2006).

Glucocorticoids are strong anti-inflammatory drugs that can also block other immune responses. Glucocorticoids medications, such as prednisone, cause reduction of inflammation and pain, and slow joint damage in RA. Side effects of these drugs may include thinning of bones, cataracts, weight gain, and diabetes. Because of their long-term side effects, they should be taken only for a short time and in low doses when possible. The using of glucocorticoids during the flare-ups of disease can lead to rapid improvement of symptoms whereas other drugs such as DMARDs have a slow onset of action.

The American College of Rheumatology Subcommittee on Rheumatoid Arthritis (ACRSRA) recommends that patients with suspected RA be referred within three months of presentation for confirmation of diagnosis and initiation of treatment with a DMARD such as methotrexate. Other DMARDS can be added or used in place of methotrexate as clinically warranted (Saag *et al*., 2008; Singh *et al*., 2012). However,

DMARDs also produce various adverse effects include suppression of immune system (Table 1.2).

Biological agents are used when arthritis is uncontrolled or toxic effects arise with DMARDs (Atzeni *et al*., 2013). These drugs design to affect parts of the immune system that plays a role in the disease process of RA. However, people taking these drugs must be observed very closely because of serious risk factors including infections from bacteria, viruses, and fungi, leukemia, lymphoma or psoriasis. The list of biological agents used for treatment of RA and their adverse effects are shown in Table 1.3.

Table 1.1 Summary of drugs used in RA treatment (Akil *et al.*, 1995)

Drug	**Mechanism**	**Adverseeffects/ disadventages**
NSAIDs	Inhibit PG synthesis	GI side effects, CNS and CVS side effects, prolonged bleeding time, exacerbation of allergic rhinitis and asthma, renal toxicity, hypersensitivity reaction
Low dose glucocorticoids	Inhibit signs and symptoms of inflammation	High blood glucose, weight gain, fluid retention, high blood pressure, osteoporosis, peptic ulcer, infection
DMARDs	Reduce inflammation, prevent joint erosions and progressive deformity	Toxicity, slow acting, expensive
Biological agents	Inhibit pro-inflammatory cytokines cascade, rapidly and significantly affect signs and symptoms, slow progressive damage to articular structures	Increase risk of infections, caution in patients with acute and chronic infections, heart failure, very expensive
Immunosuppressive drugs	Suppress immune system	Toxicity

Table 1.2 Mechanisms of action and adverse effects or disadvantages of various DMARDs (Akil *et al.*, 1995; Singh *et al.*, 2012)

Drug	**Mechanism**	**Adverseeffects/ disadventages**
Methotrexate	Interferes with folic acid synthesis, inhibits proliferating inflammatory cells	GI distress, hepatotoxicity, bone marrow suppression
Sulfasalazine	Unknown	Bone marrow toxicity, hepatitis, allergic myocarditis, hemolytic anemia, exfoliative dermatitis
Hydroxychloro-quine	Inhibits locomotion of neutrophils and chemotaxis of eosinophils	Dermatitis, cardiomyopathy, hematologic abnormalities, visual disturbances
Gold salts (e.g., auranofin)	Reduce migration of macrophage phagocytosis and cause stabilization of lysosomes	Rash, leukocytopenia, proteinuria, pruritus, persistent diarrhea
D-penicillamine	Hypothesized to inhibit T helper cell function, decreases circulating IgM rheumatoid factor	GI distress and intolerance, autoantibodies, neurologic symptoms, vasculitis

Table 1.3 Mechanisms of action and adverse effects or disadvantages of various biological agents (Atzeni *et al*., 2013; Corrado *et al*., 2013)

Drug	**Mechanism**	**Adverse effects/disadventages**
Infliximab (Remicade)	Neutralizes TNF-α, thus suppressing TNF-induced proliferation of immune cells and macrophages	Hypersensitivity reactions, serious infection, heart failure, increased risk of cancer
Etanercept (Enbrel)	Recombinant form of TNF receptor that binds circulating TNF	Hypersensitivity reaction, serious infections, increased risk of cancer
Adalimumab (Humira)	Monoclonal antibody for TNF-α	Serious infections, increased risk of cancer, lupus-like syndrome
Golimumab (Simponi)	Binds to both soluble and transmembrane bioactive forms of TNF-α	Serious infections, redness, itching, pain and swelling at the injection site, liver enzyme elevation
Certolizumab (Cimzia)	Neutralizes membrane-associated and soluble human TNF-α	Serious infections, heart failure**,** nervous system problems, allergic reactions, reactions at the injection site
Anakinra (Kineret)	Antagonized IL-1 receptor	Infection, headache, GI distress
Tocilizumab (Actemra)	Inhibits IL-6 mediated signaling through IL-6 receptors	Serious infections, infusion reactions, GI perforations, anaphylactic reactions
Abatacept (Orencia)	Blocks CD80&CD86 Co-stimulation, disrupts the interaction of antigen presenting cells with T cells	Serious infections, headaches, common colds, sore throat, nausea, allergic reactions
Rituximab (Rituxan)	depletes B-cells through complement-mediated lysis, Ab-dependent cellular cytotoxicity, induction of apoptosis	Serious infections**,** heart, stomach and kidney problems**,** serious bowel problems, low blood cell counts

1.5 Gastric ulcer

Gastric ulcer is a type of peptic ulcer disease (PUD) which occurs in the GI tract. The pathogenesis of gastric ulcer results from an imbalance of defensive mucosal barrier function and aggressive gastric luminal factors (Malfertheiner *et al*., 2009). The defensive factors are mucosal blood flow, surface epithelial cells, PGs, phospholipids or surfactant, mucus, bicarbonate secretion, gastric motility, mucosa impermeability against H^+ ion, heat shock protein, and others. The aggressive factors are gastric juice acid, pepsin, bile reflux, *Helicobacter pylori* bacterium, alcohol, and NSAIDs (Syam *et al*., 2009). Among these, the most important factors are *H. Pylori* infection and NSAIDs usage (Hawkey, 1996; Hippisley-Cox *et al.*, 2005; Papatheodoridis *et al.*, 2006; Ramakrishnan *et al*., 2007; Musumba *et al.*, 2009; Marcus *et al.*, 2013).

NSAIDs are widely used in many patients to relieve inflammation, especially in chronic inflammatory diseases such as OA and RA. Prolonged use of these drugs may produce many side-effects such as gastric erosion, peptic ulcer, and bleeding (Musumba *et al*., 2009). As shown in Figure 1.3, the pathophysiology of NSAIDs- induced gastric injury depends on their ability to decrease PGE_2 production through COX inhibition. An other mechanism is COX-independent mechanism (Musumba *et al*., 2009). The combination of COX-dependent and COX-independent mechanisms leads to oxidative tissue injury, which play a major role in the pathogenesis of NSAIDs-induced gastric damage (Hiraishi *et al*., 2000; Maity *et al*., 2009)

The annual incidence of NSAIDs associated upper GI complications such as bleeding is approximately 1–1.5%; and reductions in these complications have been demonstrated with misoprostol, proton pump inhibitors (PPIs), and COX-2 selective inhibitors (Laine *et al*., 2008).

Therefore, the searching for medicinal plants that possess anti-inflammatory activity without mucosa side effect will be a valuable substitute agent for patients suffering from chronic inflammatory disease especially RA.

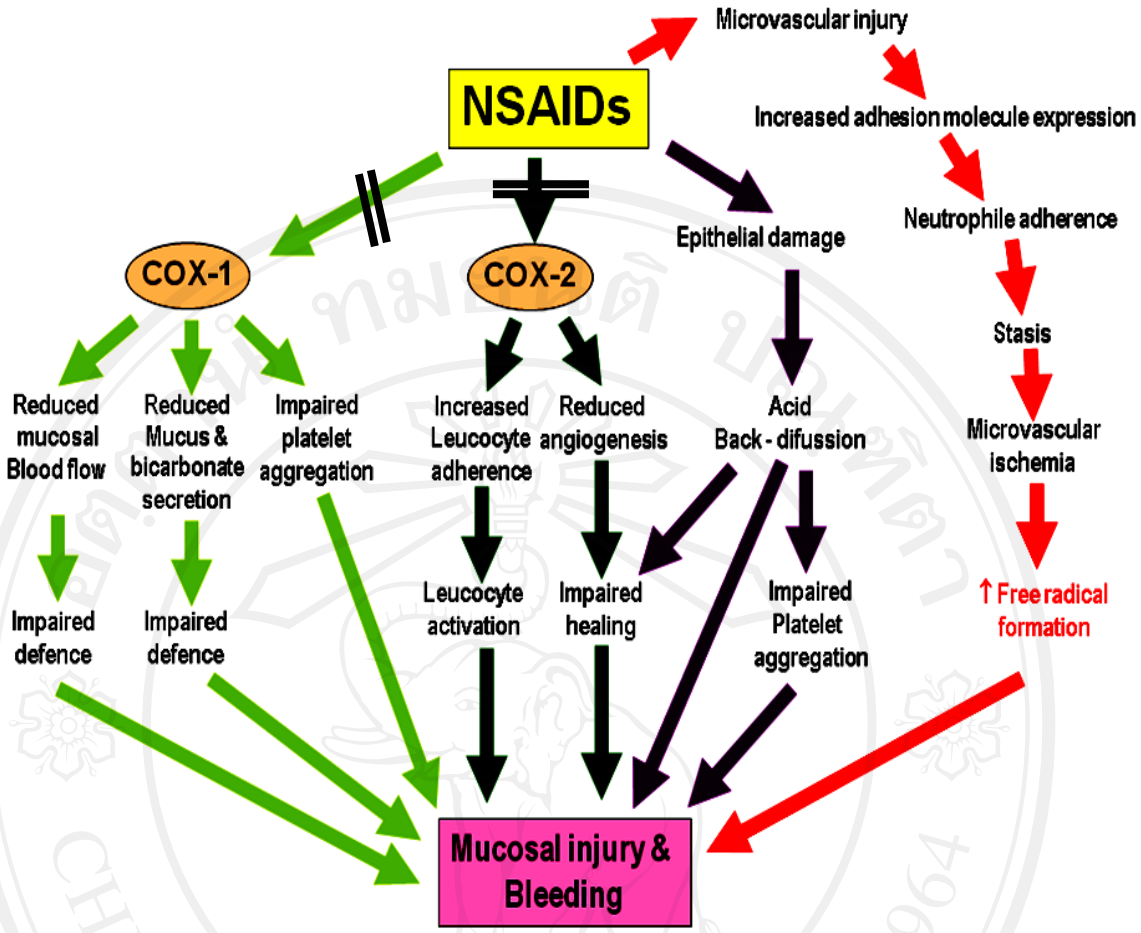

Figure 1.3 Pathogenesis of NSAID-induced gastric injury and bleeding (Musumba *et al*., 2009).

1.6 Literature review

Herbs for inflammatory diseases

Herbs and spices are now widely used in the tropical countries for many purposes including to relieve the pathogenesis of many inflammatory disorders. Medicinal plants such as *Garcinia mangostana* has been used for many years as a herbal medicine for skin infections, wounds, and diarrhoea in Southeast Asia. The xanthones from *G. mangostana*, α- and γ-mangostins, have been reported to exert anti-inflammatory activity. The mechanisms for anti-inflammatory activity of this plant is through inhibiting NO and PGE_2 production (Chen *et al*., 2008). Tumeric is a spice that comes from the root of *Curcuma longa*, a member of the ginger family, Zingaberaceae. It has a long history of use in Ayurvedic medicine for the treatment of inflammatory conditions (Jurenka, 2009). Curcumin, the constituent of tumeric, has been reported to mediate anti-inflammation by inhibiting the molecules involved in inflammation including phospholipase, lipoxygenase (LOX), COX-2, leukotrienes (LTs), thromboxane, PGs, NO, collagenase, elastase, hyaluronidase, monocyte chemotactic protein-1 (MCP-1), interferon-inducible protein, TNF, and IL-12 (Chainani-Wu, 2003). Some herbs are suggested to be used for decreasing inflammation and painful swelling in arthritis. It has been suggested that curcuminoids may be effective in preventing RA since it shows anti-arthritic effect in rats induced by streptococcal cell wall (SCW) (Funk *et al*., 2006) and complete Freund's adjuvant (CFA) (Ramadan *et al*., 2011). Harshingar (*Nyctanthes arbor tristis* Linn.) has been used for the treatment of arthritis in Indian Ayurvedic system of medicine. Water soluble ethanol extract of its leaves has been reported to reduce the levels of inflammatory cytokines (IL-1, TNF-α) in arthritic mice (Paul *et al.*, 1997).

These data support the use of these herbs in traditional and complementary medicine. Therefore, the search for herbal medicines with anti-arthritic and gastroprotective effects may primary lead to the development of new agents from natural source, which can be use in health care in the future.

***Murdannia loriformis* (Hassk) Rolla Rao et Kammathy**

M. loriformis is a herb in the family Commelinaceae. It has 1.5 cm width and 10 cm length leaf. The flower shows on the stem in blue or purple petal (Figure 1.4). It needs just a small patch of land, thrives in loose and sandy soil and under the shade, such as a large tree (Saralamp *et al*., 1996). It is originated in southern China and is abundant in northern Thailand. Its Thai name is "Ya Pak King" (หญ้าปักกิ่ง). *M. loriformis* became famous in 1984 when a patient recovered from cancer after drinking the juice of its fresh leaves. It is used for chronic bronchitis, as a remedy for cancers in early state, for treating other diseases including colds, throat infections, pneumonia, flu, and also used for wound healing (Jiratchariyakul *et al*., 2006). Many cancer patients have taken this herb to reduce side effects from radiotherapy and chemotherapy. It has been claimed to cure or prevent cancer by strengthening the immune system (Jiratchariyakul *et al*., 2006). From previous phytochemical study, *M. loriformis* contains phytosteryl glucoside (G1a), glycosphingolipid (G1b), amino acid, flavonoids, and plant membrane lipid (Jiratchariyakul *et al*., 1996; Jiratchariyakul *et al*., 1998). A cytotoxic glycosphingolipid compound, 2, *β*-O-D-glucopyranosyl- 2-(2'-hydroxy-Z-6'-enecosamide) sphingosine, is also found in the ethanol extract of *M. loriformis* (Jiratchariyakul *et al*., 2006).

Acute toxicity study in rats reveals that *M. loriformis* ethanol extract at a high dose of 5,000 mg/kg does not cause death or any abnormality (Somja, 2005). The acute toxicological evaluation from the fresh juice of this plant reveals an oral LD_{50} value greater than 120 g/kg body weight or about 300 times of human dose (Tappayuthpijarn *et al*., 1991b). Oral administration of fresh juice from *M. loriformis* does not cause abnormality in blood chemistry, CBC and pathology of internal organs. Subchronic toxicity study shows that rats which received fresh juice from *M. loriformis* in the concentrations of 2.8, 7.0 and 14 g/kg for 3 months do not show any signs of chronic toxicity (Tappayuthpijarn *et al*., 1991a). The summary of activities of *M. loriformis* is presented in Table 1.4.

M. loriformis shows anti-inflammatory, antipyretic and analgesic effects in rat models (Somja, 2005). Previous study reported that *M. loriformis* produces anti-inflammatory effect in acute and chronic inflammatory models. It significantly reduces granuloma

formation induced by cotton pellet in rats. Since this model represents chronic inflammation, therefore, it may be effective in RA disease as well. Moreover, this plant shows anti-inflammatory effect without side effect on gastric mucosa, thus it might be valuable for the long-term treatment of RA. In addition, *M. loriformis* also shows analgesic effect in pain models, i.e., acetic acid induced writhing response and formalin test (Somja, 2005). The analgesic effect of this plant may also provide benefit in RA. From our pilot study on gastroprotective effect, *M. loriformis* inhibited gastric mucosa damage induced by EtOH/HCl. Therefore, study of a potential of *M. loriformis* as a gastroprotective agent is included.

Table 1.4 The summary of activities of *M. loriformis*

Activity	References
Anti-inflammatory, antipyretic, analgesic activities	Somja, 2005
Aflatoxin-albumin adduct formation inhibition	Vinitketkumnuen *et al*., 1999
Antimutagenic activity	Rearungchom, 1993; Vinitketkumneun *et al*., 1996; Intiyot *et al*., 2002
Antiproliferative activity	Pornprasert *et al*., 2001; Koontongkaew *et al*., 2009
Antitumor activity	Vinitketkumneun *et al*., 1996
Chemopreventive effect	Kinouchi *et al*., 1997
Cytotoxic activity	Jiratchariyakul *et al*., 1998
Immunomodulator activity	Jiratchariyakul *et al*., 2006
T-lymphocyte stimulation	Jiratchariyakul *et al*., 2006

Figure 1.4 *Murdannia loriformis* (Hassk.) Rolla Rao et Kammathy
(Available at http://medplant.mahidol.ac.th/herb_aids/data/immune/m_loriformis.htm)

1.7 Hypothesis

The hypothesis of this study was that *M. loriformis* is a potential anti-arthritic and gastroprotective agent.

1.8 Objectives

The goals of this study were to investigate the anti-arthritic and gastroprotective activities of the ethanol extract of *M. loriformis.* The possible mechanisms mediated the anti-arthritic and gastroprotective activities of the ethanol extract of *M. loriformis* were also examined.

1.9 Research designs and methods

1.9.1 *In vitro* anti-inflammatory models

1.9.1.1 LPS stimulated nitric oxide production in RAW264.7 cells

RAW264.7 cell is a mouse macrophage cell line. During inflammation, macrophages play a central role in managing many different immunopathological phenomena, including the overproduction of pro-inflammatory cytokines and inflammatory mediators (Weon-Jong *et al*., 2009). Lipopolysaccharide (LPS) is a component of the outer membrane of gram-negative bacteria. The stimulation of RAW264.7 cells with LPS via Toll-like receptor 4, causes transcription of pro-inflammatory cytokines and inflammatory mediators such as IL-1ß, IL- 6, COX-2, TNF-α and iNOS. The activation of iNOS leads to subsequent NO production (MacMicking *et al*., 1997).

1.9.1.2 Quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay

There are many methods for quantifying gene expression such as Northern blotting, *in situ* hybridization, RNase protection assays, cDNA arrays, and reverse transcription polymerase chain reaction (RT-PCR). Quantitative real time polymerase chain reaction (RT-qPCR) or kinetic PCR is a

laboratory technique based on the polymerase chain reaction. The RT-qPCR is a method used to quantify the mRNA of cytokines, which are expressed at low levels (Bustin, 2000; Liu *et al.*, 2002). RT-qPCR technique is very accurate and sensitive, allows a high throughput and can be performed on very small samples and it is the method of choice for quantification of cytokine profiles in immune cells or inflamed tissues (Giulietti *et al*., 2001).

1.9.2 *In vivo* models

1.9.2.1 Complete Freund's adjuvant (CFA)-induced arthritis

CFA-induced arthritis is a useful tool to study the pathogenic process of RA (Hegen *et al*., 2008). CFA-induced arthritis shares many features with human RA including genetic linkage, synovial $CD4^+$ cells and T cell dependence (Goodson *et al*., 2003; Durai *et al*., 2004). This model is used to investigate anti-inflammatory and anti-arthritic agents and for evaluation of the anti-inflammatory properties of new drugs (Singh *et al.*, 1996). CFA was injected into the right hind paw, the positive results i.e. the swelling of the left paw are found on the day 14^{th} up to the day 42^{nd} (Castell *et al*., 1985; Castell *et al*., 1986).

1.9.2.2 Ethanol/hydrochloric acid (EtOH/HCl)-induced gastric lesions

The gastric lesion produced by EtOH resulting from its necrotizing action which causes a reduction of gastric mucosal defensive factors (Glavin *et al.*, 1992). The combination of HCl and EtOH causes the diffusion of acid into the gastric mucosal, resulting in increasing tissue injury and deepening the necrosis (Wallace, 2008). The EtOH/HCl ulcer model is commonly used for determination of the anti-gastric ulcer activity that involves the effects on gastric mucosal protective factors. The ethanol-induced lesions depend on the dose of ethanol and can be prevented by cytoprotective agent (Araki *et al*., 2000). It has been reported that histamine 2 (H_2)-receptor antagonists (e.g., cimetidine, ranitidine), can prevent the gastric lesions in this model,

suggesting that these drugs have a cytoprotective activity and partly to their ability to suppress acid secretion (Miyata *et al*., 1991). Antioxidant agents have been also reported to be effective in this model (Repetto *et al.*, 2002; Kanter *et al.*, 2005).

1.9.2.3 Indomethacin-induced gastric lesions

Indomethacin causes gastric ulceration by inhibiting COX enzymes resulting in decreasing cytoprotective PGs production (Vane, 1971; Selling *et al.*, 1987; Hayllar *et al.*, 1995; Hawkins *et al.*, 2000). The inhibition of COX pathway leads to over production of LTs and other products of 5-LOX pathway (Rainsford, 1987). It has been reported that, LTC_4 mediate gastric mucosal damage by 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). The gastric lesions in this model are prevented by PPIs (Blandizzi *et al*., 2005; Cavallini *et al*., 2006), acid anti-secretory agents such as H_2-receptor antagonists (Kuratani *et al*., 1992), cytoprotective agents (Wilson, 1987; Cavallini *et al*., 2006) and antioxidant agents (Ohta *et al*., 2006; Bhattacharya *et al*., 2007).

1.9.2.4 Restraint water immersion stress-induced gastric lesions

Restraint water immersion stress-induced gastric ulcer has been widely accepted for studying stress ulcer (Takagi *et al*., 1964a; Uramoto *et al*., 1990). This model mimics clinical acute gastric ulcerations caused by trauma, surgery, or sepsis (Ernst *et al*., 1998). The pathogenesis mechanisms of stress induced gastric mucosal lesions include, disturbance of gastric mucosal microcirculation (Kitagawa *et al*., 1979; Hemmer *et al*., 1980; Murakami *et al*., 1985), increase gastric secretion (Brodie *et al*., 1962; Kitagawa *et al*., 1979; Murakami *et al*., 1985) and abnormal gastric motility (Watanabe, 1966).

It has been suggested that the lesions in restraint water immersion stress-induced ulcer is resulted from parasympathetic overactivity (Xie *et al*., 2005). Pretreatment of animals with atropine or vagotomy, inhibits the

increase in acid output and also inhibits the ulcer formation in this model (Kitagawa *et al*., 1979). The stress-induced gastric ulceration is a consequence of the generation of reactive oxygen species (ROS) leading to oxidative damage of the gastric mucosa (Das *et al*., 1997; Kwiecien *et al*., 2002). It has been reported that lipid peroxidation may play a role in the pathogenesis of gastric mucosal lesions induced by stress (Shian *et al*., 2000).

1.9.2.5 Gastric visible mucus secretion

Surface epithelial cells are capable of secreting mucus and HCO_3^-, both of which are important in gastric defense (Flemstrom *et al.*, 1984). The mucus gel layer is able to protect the gastric mucosa from acid, pepsin, and mechanical damage (Holt *et al.*, 1986). In the gastric visible mucus assay, the EtOH/HCl is used as a necrotizing agent to destroy the gastric wall mucus in rats. Cytoprotective drugs such as synthetic PGs and sucralfate can prevent mucosal damage in this model (Lacy *et al.*, 1982; Tarnawski *et al.*, 1985; Reimann *et al.*, 1987; Gaudio *et al.*, 1993).

1.9.2.6 Pylorus ligation

The pylorus ligation model has been first described by Shay (Shay, 1945). Ligation of the pylorus is a powerful method for stimulating gastric acid secretion in rats. The accumulation of intraluminal HCl leads to auto-digestion of the gastric mucosa and break down of the gastric mucosal barrier (Sairam *et al*., 2002). This model is used as a screening method of gastric anti-secretory and antipeptic ulcer agents. It has been reported free radicals are involved in the elevation of acid and pepsin content in the gastric juice of pylorus ligated rats (Rastogi *et al*., 1998).