CHAPTER 4

4.1 DISCUSSION

Inflammation is the body reaction to invasion by infectious agent, antigen challenge or even just physical, chemical or traumatic damage (13-15). It is a protective response intended to eliminate the initial cause of cell injury. Inflammation accomplishes its protective mission by diluting, destroying or otherwise neutralizing harmful agents. It is established that cyclooxygenase (COX) is the enzyme that catalyses the conversion of arachidonic acid to prostaglandins (PGs) and tromboxane. COX was demonstrated to exist as two distinct isoforms. COX-1 is constitutively expressed as a "housekeeping" enzyme. It regulates several homeostatic processes such as renal blood flow, gastric cytoprotection and platelet aggregation, while COX-2 is the enzyme responsible for generation of most of the inflammatory PGs (53, 54). COX-2 can be up-regulated by various pro-inflammatory agents, including lipopolysaccharides, cytokines, and growth factors (121). Arachidonic acid can also be converted to leukotrienes (LTs) by the action of 5-lipoxygenase (5-LOX). LTs are potent mediators of inflammation; LTC_4, LTD_4, and LTE_4 are potent bronchoconstrictors, whereas LTB_4 is chemotactic for leukocytes. These LTs also induce the synthesis and release of other pro-inflammatory mediators (59). Inflammatory diseases are currently treated with steroidal and non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs exert their effects by inhibiting the metabolism of arachidonic acid via cyclooxygenase and some also via lipoxygenase enzyme pathways. Despite their widespread use, NSAIDs are often associated with severe adverse effects such as gastrointestinal ulceration and bleeding, and platelet dysfunctions (110, 121, 122). Many of the side effects of NSAIDs are caused by a suppression of COX-1 activity, whereas inhibition of COX-2-derived prostanoids facilitates the anti-inflammatory, analgesic, and antipyretic effects of NSAIDs. For this reason, safer compounds which have anti-inflammatory, analgesic, and antipyretic effects but less side effects are needed.

Murdannia loriformis (Hassk) Rolla Rao et Kammathy is a herb in the family Commelinaceae (86). Many plants in this family have been used as folk medicines for wound healing, diuretic and astringent properties (87). Chinese practitioners use *M. loriformis* as a remedy for cancers in early state, as well as for treating other diseases including colds, throat infections, pneumonia, diabetes mellitus (88), flu (88, 89), and facilitating wound healing (105). In Thailand, it was used by cancer patients to reduce side effects from chemo- and radiotherapy, and to stimulate the immune response (88, 89). Many studies revealed that it is effective as an anticancer, antioxidant and anticarcinogenic enzyme induction (86, 87, 89, 98-103). There is a close relationship between cancer and inflammation (110, 111, 116). Many studies suggest that COX-2-derived prostaglandins may play an important role in tumor viability, growth, and control of metastasis (114). The study in human with prostate cancer shows a relationship between COX-2 expression and the local chronic inflammation within prostate cancer and the increased angiogenesis (113). COX-2 probably is a gene involved early in carcinogenesis of cervical carcinoma by increase of PGs, and accelerates the progress of tumor by increase of PGs and vascular endothelial growth factor (VEGF) (112). Celecoxib treatment inhibited COX-2 activity and caused significant growth arrest in breast cancer cell lines (117). Multiple studies have shown that nonselective COX and selective COX-2 inhibitors effectively prevent experimental colon cancer (109). Furthermore, sulindac and the selective COX-2 inhibitor celecoxib, were shown to regress colorectal polyps in patients with familial adenomatous polyposis (109). *Murdannia loriformis* has always been used by patients with various cancer types (86-89, 91) and it shows anticarcinogenic effect in the *in vitro* studies (92, 95, 98-103). From the close relationship between cancer and inflammation mentioned above, this plant may also be potential as an anti-inflammatory agent. Preliminary screening of the ML extract for topical anti-inflammatory activity showed that, it exhibited marked inhibitory effect on the ear edema formation induced by ethyl phenyl propiolate (EPP) (108). It is well known that to investigate the effects of drugs on the acute phase of inflammation, models induced by pro-inflammatory agent such as carrageenan, dextrane,

formaldehyde, serotonin, histamine and bradykinin in rat paw are employed. In this study, acute anti-inflammatory effects of the ML extract was investigated by using carrageenin and AA-indued paw edema in rats.

Carrageenin-induced inflammation, the most widely used, primary test for screening new anti-inflammatory agents measures the ability of a compound to reduce local edema induced in the rat paw by injection of an irritant agent (66,69). The development of edema in the paw of the rat after the injection of carrageenin has been described as biphasic events (67). The first phase seen during the 1.5 h, is caused by the release of histamine and serotonin, and subsequently followed by the release of bradykinin from 1.5 to 2.5 hour. The second phase, the mediator of which is suspected to be prostaglandins occurs from 2.5 - 6 hour after carrageenin injection (67, 123-125). The second phase of swelling is due to the release of prostaglandin-like substances (67). It had been reported that the second phase of edema is sensitive to most clinically effective anti-inflammatory drugs (67, 69). This test is excellent for detecting inhibitors of COX (70). In the present study, the results obtained from the rat paw edema model show that indomethacin, a COX–inhibitor markedly reduced the paw edema after carrageenin injection. It seems to block all stages of the acute inflammation. Oral administration of the ML extract significantly reduced the edema formation of the rat paw from 1 h after edema induction to the last assessment time of 5 h. This extract showed significant edema inhibitory effect, suggesting that its main mechanisms of action may involve inhibition of prostaglandin biosynthesis. Furthermore, ML extract may influence other inflammatory mediators e.g. histamine, serotonin, and pro-inflammatory cytokines which are released after carrageenin injection. Ueno *et al*. (2000) demonstrated that bradykinin released by carrageenin may be a key mediator to induce PGI_2 formation, and both autacoids work together to induce enhanced inflammatory exudation (126). There are several lines of evidence to support the notion that COX-derived products are important mediators of inflammation, i.e., PGs synthesis is increased at the sites of inflammation (32). The major metabolites of the COX pathway are PGI_2, PGE_2 and $PGF_{2\alpha}$, which cause vasodilation and edema formation (51, 52). The results in this test

model support the possible mechanism of action of the ML extract on the COX pathway and on other inflammatory mediators, which are involved on paw edema caused by carrageenin (69).

The current therapeutic approach and chemical design of NSAIDs are targeted to developing selective COX inhibitors. However, products generated by 5-LOX pathway are particularly important in inflammation; indeed, LTs increase microvascular permeability and are potent chemotactic agents, in particular LTB_4, which is involved in leukocyte recruitment at the site of injury, also contributes and sustains the inflammatory process at the site of the injury (59, 127). Inhibition of 5-LOX indirectly reduces the expression of TNF-α (a cytokine that plays a key role of inflammation) (127). AA-induced paw edema model, was therefore also used for studying the anti-inflammatory effect of ML extract. Metabolism of AA via the lipoxygenase pathway is necessary for production of edematous response. Leukotriene products cause edema together with increase microvascular permeability (71). This test is sensitive to dual inhibitors of AA metabolism (such as phenidone), lipoxygenase inhibitors and corticosteroids, but insensitive to selective cyclooxygenase inhibitors (72).

In the present study, it was found that phenidone (40 mg/kg), the dual blocker markedly inhibited AA-induced paw edema. The ML extract at the dose of 400 mg/kg showed significant reduction of the paw edema induced by AA as same intensity as phenidone. In contrast, indomethacin (10 mg/kg), a COX inhibitor did not show any effect on this model. The results obtained from this model showed the anti-inflammatory effect of ML extract in acute inflammation via the LOX pathway. The mechanisms of the extract may depend on the inhibition of the formation and/or release of several inflammatory mediators. The findings from both paw edema models suggest that the mechanisms of action of ML extract are related to the inhibition of both the COX and the LOX pathways.

The ML extract also exerted its effect in chronic inflammatory model. Chronic inflammation is a reaction occurs when the acute response is insufficient to eliminate pro-inflammatory agents (128). The inflammatory granuloma is a typical feature of

established chronic inflammatory reaction (129). Implanting a foreign body under the skin is used to study the effect of a drug on the proliferative phase of inflammation. The response to subcutaneously implanted cotton pellet in rat has been divided into three phases: transudative phase, exudative phase and proliferative phase (73). The fluid absorbed by the pellet greatly influences the wet weight of the granuloma, and the dry weight correlated well with the amount of granulomatous tissue formed (73, 130). The granuloma formed by day 7 is characterized by the formation of a vascularized fibrous capsule containing fibroblasts and infiltrating mononuclear cells (130-132).

The results on the cotton pellet-induced granuloma formation revealed that prednisolone at a dose of 5 mg/kg significantly decreased the transudative weight and the granuloma formation. Indomethacin (5 mg/kg) appeared to be effective in inhibiting the transudative and granuloma weight on this model as well. It has been reported that corticosteroids effectively inhibit proliferative phase of inflammation during the early phase of granuloma development at the level of cell infiltration and also inhibit fibroblast proliferation (73, 131). The reduction in transudative weight by corticosteroids and NSAIDs are mainly due to decreased production of vasodilator PGs and helps in maintaining blood volume by decreasing vascular permeability (133). Indomethacin causes a decrease in granuloma tissue arising as a result of cellular reaction by inhibiting granulocyte infiltration to foreign body implanted (134). ML extract at the dose of 400 mg/kg significantly reduced the granuloma formation and transudation, although this effect was not so strong as those of the reference drugs. The results obtained suggest that ML extract inhibited the proliferative phase of inflammation, by decreasing the cellular migration to injured sites and the fibroblast proliferation as well as inhibiting vasodilator PGs synthesis.

Prednisolone markedly reduced the thymus weight and slightly but significantly reduced the body weight gain when compared with those of the control group. Although corticosteroids, such as prednisolone, stimulate protein synthesis in liver, they have pronounced catabolic effects on lymphoid and connective tissue, muscle, fat and skin. The loss of the body weight gain and the thymus weight in long term prednisolone

treatment may be due to protein catabolism and lymphoid tissue destruction, respectively (135). Indomethacin and ML extract had no effects on the body weight gain and the thymus weight. These results revealed that the ML extract possesses non-steroidal like anti-inflammatory effect.

During chronic inflammation, leukocytes always migrate to the site of injury. They accumulate at sites of inflammation and release lysosomal enzymes and oxygen radical (136). It is known that the lysosomal enzymes such as alkaline phosphates activity in serum and in the exsudate elevate during inflammation. This elevation can be normalized by both NSAIDs and steroidal drugs via the stabilization of lysosomal membrane and Inhibition of the migration of inflammatory cells into the site of inflammation (136, 137). In the present study, it was found that indomethacin, prednisolone, and ML extract could normalize alkaline phosphatase activity in rats in cotton pellet-induced granuloma model. This effect of ML extract may also result from stabilization of the lysosomal membrane and inhibition of the migration of inflammatory cells into the site of inflammation similar to NSAIDs and steroidal drugs.

The mechanism of action of ML extract in cotton pellet-induced granuloma model seems to be similar to that of NSAIDs, but anyhow it did not show gastro-irritating effect. The comparison of ulcerogenic activity between ML extract-treated rats and reference drugs-treated rats revealed that ML extract did not produce any ulcer and the gastric mucosa was found to be normal similary to that of the control group. On the contrary, indomethacin and prednisolone produced marked ulcerogenic activity. The stomach of indomethacin-treated rats showed pale color and thin wall when compared with that of the control group. COX-1 is expressed in gastric mucosa and mediates a “housekeeping” function (41, 51). The inhibition of COX by NSAIDs results in gastric ulceration (62). Highly selective COX-2 inhibitors, celecoxib and rofecoxib which were designed to relieve pain, fever, and inflammation, are as effectively as older NSAIDs, but with fewer adverse effects, especially stomach damage (64). The anti-inflammatory action of ML extract can therefore be postulated to have a selective inhibitory effect on COX-2. Several experimental data support the postulation that COX inhibition by

NSAIDs, besides causing a reduction in the synthesis of vasodilatory and gastroprotective PGs, diverts arachidonate to the 5-LOX pathway, thus increasing the formation of LTs and cysteinyl-LTs (138). These eicosanoids cause vasoconstriction of the gastric mucosa and increase the formation of reactive oxygen radicals from the peroxydative cleavage of hydroxyeicosatetraenoic acids, with further mucosal injury (127). Since ML extract showed inhibitory effect in AA-induced rat paw edema, indicating that it inhibited the LOX pathway. Non-ulcerogenic property of this extract may be also due to its inhibitory effect on LTs and cysteinyl-LTs synthesis. In addition, ML extract is a crude extract, which contains numerous compounds. Some constituents in ML extract may have cytoprotective effect and can protect the mucosa from irritating substances. The anti-inflammatory without ulcerogenic effect is a clinical desirable characteristic of novel anti-inflammatory agents, and ML extract meets this point.

Prostaglandins are potent hyperalgesic mediators which modulate multiple sites along the nociceptive pathway and enhance both transduction (peripheral sensitizing effect) and transmission (central sensitizing effect) of nociceptive information (139). Formalin test in mice is a very useful method for not only accessing the antinociceptive drugs but also helping in elucidation of the mechanism of pain and analgesia (80, 140). This model is sensitive for various classes of analgesic drugs (79). The formalin test is contributed by two distinct phases, the early phase lasting the first 5 min and the late phase lasting from 20-30 min after formalin injection (78). It is suggested that the early phase is due to a direct effect on nociceptors C-fiber (non-inflammatory pain) (78, 79, 81). This phase can be inhibited by centrally acting analgesics such as morphine and codeine. The late phase seems to be an inflammatory response in peripheral tissue and functional changes in the dorsal horn of the spinal cord (inflammatory pain) (78, 79, 81). This phase can be inhibited by NSIADs and steroids as well as centrally acting drugs (79).

The results in this study show that codeine at the dose of 50 mg/kg caused marked reduction of licking time in both phases. Opioids exert their actions by interfering pain transmission in the central nervous system (CNS) (141). Inflammation

causes the induction of COX-2, leading to the release of prostanoids, which sensitize peripheral nociceptor terminals and produce localized pain hypersensitivity (142). Indomethacin (10 mg/kg) and ML extract at the doses of 20, 40 and 80 mg/kg also produced inhibitory effect on both phases of this model. It has been generally accepted that in the late phase NSAIDs prevent the development of inflammation and produce their analgesic effects by blocking the synthesis of prostaglandins in the periphery (141). The effect of ML extract in the late phase may also be due to its inhibitory effect on inflammatory reaction in the peripheral tissue by reduction of the synthesis and/or release of PGs and other inflammatory mediators.

Within the past few years it has become increasingly clear that, apart from sensitizing peripheral nociceptors, PGs may also act in the central nervous system to produce hyperalgesia (121, 143). COX-2 is expressed constitutively in the dorsal horn of the spinal cord and becomes up-regulated briefly after trauma, such as damage to a limb, in the corresponding sensory segments of the spinal cord (144). In the spinal cord nociceptor signals are transferred to secondary neurons, which propagate the signals to the higher centers of the CNS. The sensation of pain is then assembled in the cortex (121). Analgesic action in the early phase of indomethacin and ML extract may be resulted from reduction of PGs production in the spinal cord. Shibata *et al*. (1989) reported that substance P and bradykinin participate in the manifestation of the early phase responses, and histamine, serotonin, prostaglandin, and bradykinin are involved in late phase responses (80). Other studies reported that nitric oxide participates in the transmission of noxious afferent messages within the dorsal horn of the spinal cord following peripheral inflammation (145, 146). These mediators take part in the inflammatory response and are able to stimulate nociceptors and produced pain (49). Based on the results of this study, it suggests that the antinociceptive effect of ML extract may be attributed to inhibition of PGs synthesis in spinal cord and peripheral tissue, and/or inhibition of other mediators involved in inducing algesia in this test model.

Regulation of body temperature requires a delicate balance between production and loss of heat, and the hypothalamus regulates the set point at which body

temperature is maintained (83). Fever is a coordinated endocrine, autonomic, and behavioral response organized by the brain in response to inflammatory stimuli. The conventional view of the steps that lead to fever production is that they begin with the biosynthesis of pyrogenic cytokines (e.g., IL-1β) by mononuclear phagocytes stimulated by the pathogens, their release into the circulation and transport to the thermoregulatory center in the preoptic area (POA) of the anterior hypothalamus then elevation of prostaglandin E_2 (PGE_2) inside the brain, affecting thermoregulatory neurons and resulting in elevation of temperature (147). IL-1β, IL-6, β-IFN, γ-IFN and TNF-α can act independently to produce fever. These cytokines are polypeptides, and it is unlikely that circulating cytokines penetrate the brain. There is evidence that they act on organum vasculosum of lamina terminalis (OVLT), and then this in turn activates the preoptic area of hypothalamus (83, 148). Cytokines are also produced by cells in CNS when these are stimulated by infection, and these may act directly on the thermoregulatory centers (83). Recently Blatteis *et al*. (2005) reviewed that LPS fever occurs in the following sequence: the immediate activation by LPS of the complement cascade, the stimulation by the component C5a of Kupffer cells, their consequent, virtually instantaneous release of PGE_2, its excitation of hepatic vagal afferents, their transmission of the induced signals to the preoptic-anterior hypothalamus. The activation of the first causes an immediate, PGE_2-independent rise in core temperature (T_c) (the early phase of fever), and of the second a delayed, PGE_2-dependent T_c rise (the late phase of fever) (149). The fever produced by cytokines is probably due to local release of PGs in the hypothalamus (83). Studies using mice with EP receptor gene deletions have indicated that the pyretic action of PGE_2 is mediated by the EP3 receptor, since mutant mice lacking this receptor do not develop fever after administration of PGE_2, IL-1, or LPS (148, 150-152).

The results in antipyretic study revealed that indomethacin at the oral dose of 10 mg/kg markedly decreased the rectal temperature within 30 min after medication. Antipyretic effect of NSAIDs is due to inhibition of the synthesis of PGs within the preoptic-anterior hypothalamus (83). ML extract at the dose of 400 mg/kg also caused reduction in rectal temperature although this effect was not so strong as that of

indomethacin. The result obtained suggests that antipyretic effect of ML extract may be resulted from inhibition of the synthesis of PGE_2.

Assessment of the acute toxicity is the first step in the toxicological investigations of an unknown substance. The method used in this study is based on the assumption that the toxicity of the extract investigated is completely unknown and in the purpose to determine the acute toxicity index (LD_{50}) with a minimum number of experimental animals as possible. Anyhow, according to OECD guidelines for testing of chemicals, if a test at one dose level of at least 5000 mg/kg body weight produces no compound-related mortality, then a full study using three levels may not be necessary (84, 85). The present result revealed that ML extract seems to be non toxic, since the high dose of the extract (5000 mg/kg) did not produce mortality or show any signs of toxicity or changes in general behavior. This extract did not causes pathological changes of the internal organs of tested animals when examination was made on the eighth day after medication.

4.2 CONCLUSION

The results obtained in the present study suggest that ML extract possesses anti-inflammatory, analgesic and antipyretic activity. The anti-inflammatory effect of ML extract was found on both acute and chronic inflammation. For acute inflammatory reaction, carrageenin and AA-induced rat paw edema models were used. ML extract significantly reduced paw edema induced by both carrageenin and AA. It seems that the extract reduces inflammatory reaction by inhibiting both the COX and the LOX pathways of arachidonic acid metabolism and/or the synthesis or release of other mediators e.g. histamine, serotonin and bradykinin.

In the chronic inflammation, ML extract reduced the transudative weight and granuloma formation, which may be due to inhibition of PGs synthesis as well as inhibition of fibroblast proliferation and cells migration to injured tissues. ML extract appears to lack of steroidal-like effects, since this extract did not cause the reduction of the body weight gain and the thymus weight. ML extract reduced the alkaline phosphatase activity in the serum, which may be due to its stabilizing effect on the lysosomal membrane of leukocytes and inhibiting granulocytes infiltration to injured site. The administration of ML extract did not cause gastric mucosal lesions when compared with indomethacin and prednisolone. Non-ulcerogenic effect of this extract may be due to its selective inhibition of COX-2 and/or inhibition of the LOX pathway and thereby reducing LTs and cysteinyl -LTs synthesis, which cause gastric mucosa injury. ML extract may contain cytoprotective components which possibly involve in gastric mucosa protection.

The analgesic and antipyretic effects of ML extract may be due to an inhibition of PGs synthesis and/or release and other mediators involved.

The rats received ML extract at the high dose of 5000 mg/kg did not cause any changes in general behavior or death; furthermore it did not produce any abnormality of the internal organs.