# CHAPTER 4

# DISCUSSION AND CONCLUSION

## 4.1 Discussion

Acute and chronic inflammatory diseases are still one of the most world important health problems (108). NSAIDs are among the most widely used of all therapeutic agents worldwide. They are frequently prescribed for rheumatic, musculoskeletal complaints and are often taken without prescription for minor aches and pain. There are now more than 50 different NSAIDs on the market and none of these is ideal in controlling or modifying the signs and symptoms of inflammation, particularly those that occur in the common and chronic inflammatory joint diseases. Virtually all currently available NSAIDs, more particularly the classical NSAIDs, can have significant unwanted effects (109) such as gastric intolerance, bone marrow depression, elevation of hepatic enzymes, water and salt retention. For these reasons, there is a need to find and develop new anti-inflammatory drugs with fewer and less serious adverse reactions (108, 109). In recent years, selective COX-2 inhibitors (e.g., celecoxib, etroricoxib, and valdecoxib, etc.), that achieve the same anti-inflammatory efficacy as classical NSAIDs but minimize the risk of unwanted side-effects, have been developed (110). The risk of serious GI events was reduced by about 50% (111-113). However, studies conducted after the introduction of COX-2 inhibitor showed an increased risk of cardiovascular adverse events. There is widespread agreement that the cardiovascular events associated with COX-2 inhibitors constitute a class effect (114). Thus, it appears that selective COX-2 inhibitors do not represent a complete answer to the need for safer and more effective drugs to be used in inflammatory disease therapy (115). In additional, the selective COX-2 inhibitors are still more expensive than nonselective NSAIDs. The investigation of the efficacy of plant-based drugs used in the traditional medicine have been paid great attention

because they are cheap, and readily available have fewer side effects (7), however, validation of their anti-inflammatory effects remain to be elucidated

*D. lomentaceum* is a herb in digineous the Northeastern part of Thailand. This plant is used by local practitioners for the treatment of pain and muscle sprain (102). The preliminary screening of anti-inflammatory property has shown that the DL extract inhibits ear edema induced by EPP in rats. It is therefore interesting to investigate the anti-inflammatory, analgesic and antipyretic activities of the DL extract in several experimental animal models. It is widely accepted that acute inflammation induced by pro-inflammatory agents such as carrageenin, AA, formaldehyde, 5-HT, histamine and bradykinin in rats are appropriate, easy and inexpensive to perform, sensitive and reliable models for evaluation of anti-inflammatory activity and mechanism of action of test drugs. In this study, acute anti-inflammatory effects of the DL extract were performed by using carrageenin and AA-induced 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 (81, 84). This test is excellent for detecting inhibitors of COX (85). Intraplantar injectin of carrageenan in the rat resuted in an increase in hind paw weight (indicative of edema) comprising a relatively rapid early phase (up to 2 h) followed by a more sustained late phase (2 h to 6 h). Previous research has suggested that the early phase inflammation is triggered by the concerted release of histamine, bradykinin and 5-HT at the inflamed site (84) whilst the late phase response is due primarily to the *de novo* formation of pro-inflammatory prostanoids and NO (116-118).

In the present study, the results obtained from the rat paw edema model show that diclofenac, a COX–inhibitor markedly reduced the paw edema after carrageenin injection. It seems to block all stages of the acute inflammation. Oral administration of DL extract significantly reduced the edema formation of the rat paw from 1 h after edema induction to the last assessment time of 5 h. The significant edema inhibitory effect of DL extract at the $1^{st}$, $3^{rd}$, and $5^{th}$ h after edema induction, suggests that its mechanisms of action may involve the PG biosynthesis and/or release. Furthermore, DL extract may influence other inflammatory mediators e.g. histamine, 5-HT, NO and

pro-inflammatory cytokines which are released at 1st h 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 (119, 120). The major metabolites of the COX pathway are $PGI_2$, $PGE_2$ and $PGF_{2\alpha}$, which cause vasodilation and edema formation (57, 59). The results in this test model support the possible mechanism of action of the DL extract on the COX pathway and on other inflammatory mediators, which are involved on paw edema caused by carrageenin.

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. The involvement of LOX products, particularly LTs, and mast cell mediators in the edematous response to AA render the AA-induced paw edema model potentially useful for studying anti-inflammatory agents with a mechanism of action different from that of COX inhibitors (87). 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 (121, 122). Inhibition of 5-LOX indirectly reduces the expression of TNF-$\alpha$ (a cytokine that plays a key role of inflammation) (121). AA-induced paw edema model, was therefore also used for studying the anti-inflammatory effect of DL extract. This test is sensitive to dual inhibitors of AA metabolism (such as phenidone), LOX inhibitors and corticosteroids, but insensitive to COX inhibitors (87).

Prednisolone (5 mg), a phospholipase $A_2$ inhibitor markedly inhibited AA-induced paw edema. In contrast, diclofenec (10 mg/kg), a potent COX inhibitor (123) did not show any effect on this model. DL extract also did not show inhibitory effect on AA-induced hind paw edema. The results obtained from this model indicates that the anti-inflammatory effect of DL extract did not mediated via the LOX pathway. The findings from both paw edema models suggest that the mechanism of action of DL extract could be related to the inhibition of the COX pathways, but is not related to the LOX pathway. It is therefore suggested that DL extract may possess more or less similar mechanism of anti-inflammatory activity as NSAIDs rather than steroids or LOX inhibitor.

Chronic inflammation is a reaction occurs when the acute response is insufficient to eliminate pro-inflammatory agents (124). The inflammatory granuloma is a typical feature of established chronic inflammatory reactions (125). The cotton pellet granuloma method is widely used to evaluate the transudative and proliferative components of chronic inflammation (126, 127). 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 in to three phases: transudative , exudative and proliferative phases (88). 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 (97, 136). The granuloma formed by day 7 is characterized by the formation of a vascularized fibrous capsule containing fibroblasts and infiltrating mononuclear cells (128-130). The steroidal and some NSAIDs drugs can effectively inhibit the granuloma formation probably via their ability to interfere with the proliferative phase of inflammation (88).

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. Diclofenac appeared to be effective in inhibiting the transudative and granuloma weight on this model as well. In contrast, DL extract at the dose of 400 mg/kg did not show any effect on the transudative and granuloma formation. It has been reported that NSAIDs cause a decrease in granuloma tissue arising as a result of cellular reaction , which is released by inhibiting granulocyte infiltration to foreign body implanted, preventing generation of collagen fibers and suppressing mucopolysaccharides. The effects of steroids on chronic inflammation are more significant than on acute inflammation. These effects depend on the inhibitory function of macrophages and fibrosis (131). These reflect to a high extent their efficacy to reduce an increase in the number of fibroblasts and synthesis of collagen and mucopolysaccharide which are natural proliferative events of granulation tissue formation (132). In the present study, DL extract did not show its effect in chronic inflammation. The results indicate that this extract dose not inhibit or reduce the granulocyte infiltration, the increasing of fibroblasts, the synthesis of collagen and mucoplolysuccharide.

Prednisolone markedly reduced the thymus weight and 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 on the otherhand, also have pronounced catabolic effects on lymphoid and connective tissues, 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 (133). Diclofenac and DL extract had no effects on the body weight gain and the thymus weight. These results lend support to previous notion in paw edema experiments that DL 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 (134). It is known that the lysosomal enzymes such as alkaline phosphatase activity in serum and in the exudate are elevated 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 (134, 135). In the present study, diclofenac and prednisolone could normalize alkaline phosphatase activity in rats in cotton pellet-induced granuloma model. In contrast, DL extract did not reduce alkaline phosphatase activity to normal level. These results revealed that the DL extract did not stabilize the lysosomal membrane as did the other two test drugs.

The gastro-irritating effects of test compounds cotton pellet-induced granuloma rats was assessed. DL extract did not produce any ulcer and the gastric mucosa was found to be similar to that of the control group. On the otherhand, diclofenac and prednisolone produced marked ulcerogenic activity. The stomach of diclofenac-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 (48, 57). The inhibition of COX by NSAIDs causes gastric ulceration (81). Highly selective COX-2 inhibitors, celecoxib and etroricoxib were designed to relieve pain, fever, and inflammation, are as effective as older NSAIDs, but with fewer adverse effects, especially stomach damage (133). From these results,

it is suggested that non-ulcerogenic property of DL extract may also be due to a selective inhibitory effect on COX-2. Although the possibility of DL extract to inhibit COX-1 connot be ruled out, crude DL extract use in present study contains numerous compounds and some constituents in DL extract may have cytoprotective effects that can counteract the irritating effects produced by COX-1 inhibition. The anti-inflammatory activity without ulcerogenic effect is a clinically desirable characteristic of novel anti-inflammatory agents, and DL extract, more favorable adverse effect profiles is certainly a welcome alternative to currently available anti-inflammatory modalities .

*D. lomentaceum* is used in traditional medicine for the treatment of pain. However, fever, pain, and inflammation are all closely related as part of the body's defence mechanisms to injury (136), therefore investigation of analgesic and antipyretic effects was also performed. The formalin test was introduced by Dubuisson and Dennis (1977) and later modified for use in mice. Injection of formalin into the hind paw of animals produces a biphasic nociceptive licking and biting response consisting of immediate (early) and tonic (late) phases. The early phase reflects the direct intense stimulation of nociceptors, whereas the last phase may be associated with a release of inflammatory mediators such as PGs (91, 134, 135, 137). 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 (95, 138). This model is sensitive for various classes of analgesic drugs (94).

PGs 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 (91). 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 (93). It is suggested that the early phase is due to a direct effect on nociceptors C-fiber (non-inflammatory pain) (93, 94, 96). 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) (93, 94, 96). This latter phase can be inhibited by NSIADs and steroids as well as centrally acting drugs (94).

The results in this study as expected show that morphine at the dose of 10 mg/kg completely inhibited the licking time in both phases. Morphine exerts its actions by interfering with pain transmission in the central nervous system (CNS) (93). Inflammation causes the induction of COX-2, leading to the release of prostanoids, which sensitize peripheral nociceptor terminals and produce localized pain hypersensitivity as evidenced in the late phase (96). In this study, diclofenac undoubtedly inhibited nociceptive behaviors only during the late phase. 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 PGs in the periphery (93). DL extract produced inhibitory effect on licking time in both phases of this model although its effect was much more pronounce on the late phase. 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 (138, 139). COX-2 is expressed constitutively in the dorsal horn of the spinal cord and becomes up-regulated in the corresponding sensory segments of the spinal cord briefly after trauma, such as damage to a limb (140). In the spinal cord nociceptive 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 (138). Analgesic action of DL extract in the early phase may be the results of reduction of PGs production in the CNS as mentioned above. Shibata *et al*. (1989) reported that SP and bradykinin also participate in the manifestation of the early phase responses, whereas histamine, serotonin, PGs, and bradykinin are involved in late phase responses (104). Other studies reported that NO participates in the transmission of noxious afferent messages within the dorsal horn of the spinal cord following peripheral inflammation (141, 142). These mediators take part in the inflammatory response and are able to stimulate nociceptors and produced pain (45). Based on the results of this study from the early phase, it suggests that the antinociceptive effect of DL extract may be attributed to inhibition of local nociceptors and/or an inhibition of mediators responsible for pain induction in the CNS at hypothalamic region. The result from the late phase, DL extract also exerted its inhibitory effect on

inflammatory pain in the peripheral tissue, which may be due to reduction of the synthesis and/or release of PGs.

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 (98). Fever is a coordinated endocrine, autonomic, and behavioral responses organized by the brain in response to inflammatory stimuli. The conventional view of the steps that lead to fever production is that it begins with the biosynthesis of pyrogenic cytokines (e.g., IL-1β) by mononuclear phagocytes stimulated by pathogens, their release into the circulation and transport to the thermoregulatory center in the preoptic area of the anterior hypothalamus then lead to elevation of $PGE_2$ inside the brain, affecting thermoregulatory neurons and resulting in elevation of temperature (143). IL-1B, 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, and then this in turn activates the preoptic area of hypothalamus (98, 144). Cytokines are also produced by cells in CNS when these stimulated by infection, and these may act directly on the thermoregulatory centers (98). The fever produced by cytokines is probably due to local release of PGs in the hypothalamus (98).

Brewer's yeast-induced hyperthermia in rats was used to investigate antipyretic activity of DL extract. Diclofenac (10 mg/kg) markedly decreased the rectal temperature. Antipyretic effect of NSAIDs is due to inhibition of the synthesis and/or release of PGs within the preoptic-anterior hypothalamus (98). DL extract (400 mg/kg) also caused reduction in rectal temperature and this effect was comparable that of diclofenac. As evidenced earlier that DL extract exerted several effects similar to NSAIDs with the notable exception on gastric ulceration, it is reasonable to assume that DL extract shares identical mechanism of antipyretic action to NSAIDs to inhibit PGs synthesis and/or release in the CNS.

**4.2 Conclusion**

The results obtained in the present study suggest that DL extract possesses anti-inflammatory, analgesic and antipyretic activity. The anti-inflammatory effect of DL extract was found on acute inflammatory reactions but has no effect on the chronic inflammation. On acute phase inflammation, DL extract significantly reduced paw edema induced by carrageenin but no significant inhibition was observed in AA-induced rat paw edema. It seems likely that the extract reduces inflammatory reaction by inhibiting only the COX pathways. In the chronic inflammation, DL extract did not show inhibitory effect on the cotton pellet induced granuloma formation and did not cause the reduction of the body weight gain and the thymus weight as did prednisolone. Thus, it is unlikely that DL extract possessed similar mechanism of anti-inflammatory action as steroidal drugs. Moreover, the administration of DL extract did not cause gastric mucosal lesions when compared with diclofenac and prednisolone. Non-ulcerogenic effect may be due to its selective inhibition of COX-2 and or cytoprotective components of this extract. The analgesic effects of DL extract may be due to an inhibition of PGs synthesis and/or release in concert with other mediators involved both centrally and peripherally whereas its antipyretic effects is likely mediated centrally. The data of the present study lend concrete support to the long traditional use of *D. lomentaceum* in the treatment of pain and muscle sprain.