CHAPTER 4
DISCUSSION AND CONCLUSION

4.1 Discussion

Inflammation is one of the cored symptom pathologic features of many diseases and the inadequacy of present day drug therapy underlines the need to improve the technical feasibility of discovering safe and effective anti-inflammatory agents. The inflammatory process involves a series of events that can be elicited by numerous stimuli such as infectious agents, ischemia, antigen-antibody interactions, and thermal or other physical injury. At a macroscopic level, the response usually is accompanied by the familiar clinical signs of erythema, edema, tenderness (hyperalgesia), and pain [21]. It is a complex process and various mediators, e.g., histamine, bradykinin, serotonin, PGs, LTs, IL-1, have been reported to be involved in the development of inflammatory diseases [80]. Anti-inflammatory agents exert their effects through a spectrum of different modes of action. All NSAIDs and steroids currently available are probably able to modulate more than one mediator or cellular event involved in the inflammatory response [14].

Generally, anti-inflammatory effects may be elicited by a variety of chemical agents and that there is no remarkable correlation between their pharmacological activity and chemical structure [81]. This fact, associated with the complexity of the inflammatory process, makes the use of different experimental models essential when conducting pharmacological trials. On the other hand, the search for a safe anti-inflammatory drug that is free from gastric intolerance continues unabated and a part of such research is the evaluation of medicinal plants known to be used for the treatment of inflammatory disorders [82].

The CS extract was preliminary screened for topical anti-inflammatory activity on the ear edema formation induced by EPP. EPP-induced rat ear edema formation is a useful model for investigating the anti-inflammatory activity of test substance on

acute phase of inflammation [41]. Topical application of EPP produces an increase in the vascular permeability with leukocyte infiltration into the ears. The long-lasting inflammatory response, which provokes by EPP, is associated with a transient increases in PG production. PGs and other inflammatory mediators, which are involved in this model, such as histamine, serotonin and bradykinin, are capable of promoting vasodilation and increasing vascular permeability as well as synergistically producing edema [83].

The results of the present study showed that the CS extract exerted an inhibitory effect on the edema formation of the ear induced by EPP. The reference drug, Daflon®, which contains two flavonoids, i.e., diosmin and hesperidin, was reported to have analgesic and anti-inflammatory activities [76]. In this study, Daflon® elicited significant inhibitory activity on the edema formation. The standard anti-inflammatory agent, diclofenac, an NSAID that is commonly employed in the treatment and/or management of rheumatoid arthritis, osteoarthritis, and ankylosing spodylitis [84, 85, 86] due to its anti-inflammatory and analgesic effects [87]. It reduces inflammation, swelling and arthritic pain by inhibiting PG synthesis [88, 89, 90]. It has been reported to suppress inflammation induced by various phlogistic agent in experimental animal models [90, 91, 92].

Phytochemical studies have shown that aerial part of *C. serratum* contains β–sitosterol, 24(S)–ethyl cholesta-5,22,25-trien-3β-ol, 5-hydroxy-7,4'-dimethoxy flavone, luteolin, apigenin, scutellarien, and ursolic acid [65], stigmasterol, a quinone pigment, α-spinasterol, luteolin-7-0-glucuronide, baicalin, scutellarien 7-0-glucuronide, and a few plant acids [66]. In previous study it has been reported that flavonoids are compounds biosynthesized by most plants and has been found to possess significant activity on the inflammatory process such as those present in Daflon® [4]. The CS extract from the aerial part of *C. serratum* also contains flavonoids and other substances expected to possess anti-inflammatory and analgesic effects. The results obtained from the present study suggest the anti-inflammatory activity of the CS extract. The mechanism of this activity may be due to the inhibition of PG biosynthesis and/or of the release of other inflammatory mediators of the acute phase of inflammation of the ear edema model.

Carrageenin-induced rat paw edema is a suitable test for evaluating anti-inflammatory drugs. This test has frequently used to assess the anti-edematous effect of natural products. The hind paw edema that follows intraplantar carrageenin injection, involves a complex and time-dependent synthesis/release of a plethora of different inflammatory mediators. As in previous studies, the edema formation due to carrageenin in the rat paw is a biphasic event [50] and mediators involved in this model have been reported by Di Rosa et al (1971). The first phase seen during the first 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 h. The second phase, the main mediators of which are PGs, occurs from 2.5 to 6 h after carrageenin injection [50, 93]. PGs (E_1, E_2, F_{1_α}, and F_{2_α}) evoke increased vascular permeability in the skin of rat and human, which appears to be an indirect effect resulting from the release of vasoactive amine from mast cells. It is well established that PGs, by virtue of their activity as modulators of inflammatory responses, have a major role in inflammatory mechanism [94]. The carrageenin-induced hind paw edema in rat is known to be sensitive to COX inhibitors, but not to LOX inhibitors, and has been used to evaluate the effect of NSAIDs which primarily inhibit the COX involved in PGs synthesis [95, 96]. It has demonstrated that suppression of carrageenin-induced hind paw edema after the third hour correlates reasonably well with therapeutic effect of most clinical effective anti-inflammatory agents [93].

In the present study, the results obtained from the rat paw edema model showed that diclofenac, a COX-inhibitor, markedly reduced the paw edema after carrageenin injection. It seemed to block all stages of the acute inflammation. Oral pretreatment of animals with the CS extract and Daflon® resulted in significant inhibition of carrageenin-evoked hind paw edema. The significant inhibitory effect of the CS extract on carrageenin-induced paw edema at the third, suggests that the main mechanism of action of the CS extract might involve the PG biosynthesis and/or release. The CS extract might also influence other inflammatory mediators e.g., histamine, serotonin, and pro-inflammatory cytokines which are released during the first after carrageenin injection. The results in this model support the possible mechanism of action of the CS extract on the COX pathway and on other

inflammatory mediators, which are involved in paw edema caused by carrageenin. The substance present in *C. serratum* which could be responsible for this activity is β– sitosterol, which has been proved to possess anti-inflammatory effect [97]. Other flavonoids found in *C. serratum* might also involve in its anti-inflammatory action.

In order to clarify other mechanisms of anti-inflammatory action of the CS extract, effects of the CS extract on the LOX pathway and on phospholipase A_2 were investigated using AA-induced paw edema model in rat. The current therapeutic approach and chemical design of NSAIDs are targeted to develope 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. Inhibition of 5-LOX indirectly reduces the expression of TNF-α (a cytokine that plays a key role of inflammation) [98]. Metabolism of AA via the LOX pathway is necessary for the production of edematous response. LTs cause edema by increasing microvascular permeability [51]. AA-induced paw edema model is sensitive to dual inhibition of AA metabolism (such as phenidone), LOX inhibitors, and corticosteroids that inhibit phospholipase A_2, but insensitive to COX inhibitors [52]. The results from the present study showed that prednisolone (5 mg/kg) markedly inhibited AA-induced paw edema. However, diclofenac (10 mg/kg), a COX-inhibitor did not show any effect in this model. The CS extract at the doses of 80 and 160 mg/kg exerted significant inhibitory effect in this animal model. The results obtained from this model indicated that the anti-inflammatory effect of the CS extract on acute inflammation may also be mediated via the LOX pathway or inhibition of phospholipase A_2. The mechanisms of the extract may depend on the inhibition of the formation of several inflammatory mediators. The findings from both paw edema models suggest that the mechanism of action of the CS extract may be related to the inhibition of both the LOX and the COX pathways or phospholipase A_2. Whatever its mechanisms of action, the results obtained in this study are in line with the therapeutic use of *C. serratum* in traditional practice for the treatment of inflammation and arthritis.

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 [99]. Formalin test in mice is a very useful method for not only accessing the antinociceptive drugs but also helping elucidation of the mechanisms of pain and [54, 57] analgesia. This model is sensitive to various classes of analgesic drugs [59]. The test consists of two distinct phases that possibly reflecting different types of pain mechanism [55, 59, 100, 101, 102]. The first phase starts immediately after injection of formalin and lasts about 5 min. This is due to direct peripheral chemical stimulation of nociceptors [55, 100] that seems to be caused predominantly by C fiber activation. In this phase, the first response is evoked by the direct formalin stimulation of the nerve endings followed by SP release, and SP may play a role through cooperation with bradykinin in this phase. The second phase starts approximately 15-20 min after formalin injection and lasts for 20-40 min [58]. The second phase appears to be dependent on the combination of an inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord [57, 58]. Several chemical mediators such as histamine, serotonin, PGs and bradykinin are involved in the second phase. These mediators take part in the inflammatory response and are also able to stimulate nociceptors and induce pain [102]. The response of the early phase can be inhibited by centrally acting analgesics such as morphine and codeine. In contrast, the late phase which seems to be due to an inflammatory response is partly mediated by PGs and can be inhibited by NSAIDs (e.g., aspirin and diclofenac), corticosteroids (e.g., dexamethasone and prednisolone), as well as the centrally acting analgesics [103, 104]. The formalin test is sensitive to NSAIDs and other mild analgesics. It is now well accepted that the anti-nociceptive efficacy of NSAIDs depends not only upon the inhibition of PG synthesis at the site of injury but also on the prevention of a nociception-induced by PGs, especially PGE_2 release in the spinal cord [105, 106].

In this study, morphine at the dose of 10 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) [107]. Inflammation causes the induction of COX-2, leading to the release of prostarnoids, which sensitize peripheral nociceptor terminals and produce localized pain hypersensitivity [76]. In this study,

diclofenac (5 mg/kg) inhibited nociceptive behavior 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 [107]. Daflon® at the dose of 150 mg/kg and the CS extract at the doses of 40 and 80 mg/kg produced anti-nociceptive effects on both phases of formalin test, but markedly on the late phase. Data obtained from the early phase suggest that the slight analgesic activity of the CS extract is mediated via an inhibition on excitation of local nociceptors and/or an inhibition of mediators responsible for pain induction in the CNS at the hypothalamic region. The result from the late phase indicates that the CS extract possesses inhibitory effect on inflammatory reaction in the peripheral tissue, which could be related to the reduction of the release and/or synthesis of inflammatory mediators, especially PGs.

As drug demonstrated efficacy in the treatment of hemorrhoids such as Daflon® can constrict human vein [76, 108], therefore, the *in vitro* model using isolated human umbilical vein was performed. This study was based on the information that *C. serratum* is the famous plant which widely used for the treatment of hemorrhoid in Thai folk medicine. Phytochemical study of *C. serratum* revealed that its major constituents are the flavonoids. The flavonoids, particularly diosmin, hesperidin, and oligomeric proanthocyanidin complexes have demonstrated potential in the treatment of hemorrhoids and varicose veins [109]. These bioflavonoids exhibit phlebotonic activity, vasculoprotective effects and antagonistic effects on the biochemical mediators of inflammation [109, 110, 111]. In pervious study, diosmin reinforces venous tone by prolonging the activity of norepinephrine in the experiment on the saphenous vein strips of dog [82]. In other studies, diosmin combined with hesperidine has been shown to possess venotonic effect on human isolated saphenous vein [76, 112]. As *C. serratum* is used to treat hemorrhoid, it is postulated that its flavonoids may also possess phlebotonic activity similar to diosmin and hesperidin. Thus, isolated human umbilical vein was used in this study to confirm the traditional use of *C. serratum* in the treatment of hemorrhoid which is causes by venous insufficiency and loss of contractility of vein.

In this study, supramaximal dose of NE (15 μM) was used to test the responsiveness of human umbilical vein and expressed as 100% response. In the

present study, Daflon® at the doses of either 0.1, 0.2, and 0.4 mg/mL contracted human umbilical vein in the same magnitudes as the CS extract and at the maximal concentration used (0.4 mg/mL), both Daflon® and the CS extract exerted strong venocontraction almost equivalent to NE. It seems that the CS extract possesses similar venotonic effect as bioflavonoids in Daflon®, however, the mechanism of action of the CS extract was not elucidated in this study. The results from the present study reveal anti-inflammatory, analgesic and venoconstrictive effects of the crude extract from *C. serratum*. Its anti-inflammatory and analgesic effects could be produced by the bioflavonoids especially β–sitosterol and leuteotin. The venotonic effect of the CS extract may be postulated to be also due to the effect of both bioflavonoids or other compounds present in the extract. The results of the present study support the efficacy of traditional use of *C. serratum* as antihemorrhoidal drug in Thai folk medicine.

4.2 Conclusion

The results obtained in the present study suggest that the CS extract possesses analgesic, anti-inflammatory, and venoconstrictive effects. The anti-inflammatory effect of the extract was evidenced by the significant reduction of edema formation in the three animal models of acute inflammatory reaction including EPP-induced rat ear edema, carrageenin and AA-induced rat paw edema. It seems that the extract reduces inflammatory reaction by inhibiting both the COX and LOX pathways of AA metabolism or inhibiting phospholipase A_2 and/or the synthesis or the release of other mediators, e.g., histamine, serotonin and bradykinin. The analgesic activity of *C. serratum* was profound as shown by the significant reduction of algesic reaction in the formalin test (both early and late phases). It is likely that this plant extract possesses an analgesic effect by inhibiting both peripherally and centrally mediated nociception. In addition, *C. serratum* exerted venoconstrictive effect when tested in the *in vitro* model of human umbilical vein, suggesting that this plant possesses venotonic effect. These analgesic, anti-inflammatory, and venoconstrictive effects are considered to be beneficial for the treatment of hemorrhoids. Thus the results of this study are in line with the traditional use of *C. serratum*.