CHAPTER 4
DISCUSSION AND CONCLUSION

4.1 Discussion

Inflammation is a complex pathophysiological process in which inflammatory stimuli produce a cascade of intracellular signaling leading to the synthesis of specific mediators that in turn induce the inflammatory responses. Steroid and NSAIDs are currently the most widely used drugs in the treatment of inflammatory disorders. It has been demonstrate that aspirin and other NSAIDs inhibit COX and therefore synthesis of PGs, key components in the inflammatory process is decreased (18). However, chronic use of analgesic and anti-inflammatory drugs such as NSAIDs has been reported to produce high risk of GI bleeding (58) and cardiovascular events (15).

The analgesic activity of AP extract was evaluated using both chemical (acetic acid-induced writhing response) and thermal (tail-flick test) methods of nociception in rats. The acetic acid-induced writhing response in mice is commonly tested for detection of peripheral and central analgesic acting of drugs. The acetic acid injection causes tissue damage as well as release of several endogenous substances such as BKs, PGs and 5-HT and contributes to the process of inflammation and increased sensitivity of nociceptors. These endogenous substances sensitize peripheral nerve terminals (peripheral sensitization), leading to phenotypic alterations of the sensory neurons and increased excitability of the spinal cord dorsal horn neurons (central sensitization) (59, 60). Consequences of peripheral sensitization are a lowering of the activation threshold of nociceptors and an increase in their firing rate. These changes result in the production of hyperalgesia and allodynia associated with nociceptive chronic pain. In addition, peripheral sensitization also plays an important role in the development and maintenance of central sensitization (61). In this model, the writhing responses elicited by intraperitoneal injection of noxious chemical stimuli (acetic acid) consist of abdominal wall contraction, pelvic rotation and followed by hind limb extension (47). The results of this study showed that pre-treatment with diclofenac and various doses of AP extract significantly inhibited the writhing

response in a dose-dependent manner. To elucidate mechanism of analgesic activity involving CNS, the tail flick test was conducted.

The traditional methods for the study of thermonociception (hot-plate and tail-flick test) are to apply a constant suprathreshold heat stimulus and measure the reflex latency of nocifensive (pain-avoiding) reactions (62). The rat tail-flick test is a measure of acute cutaneous thermal pain and is generally considered to be a measure of nociceptive threshold (63). It is well-known that pain produced in the tail-flick test is sensitive to and inhibited by centrally acting analgesic drugs such as morphine and codeine, which was also evidenced in the present study. The maximal dose of AP extract (600 mg/kg) used in the present study, and diclofenac did not show analgesic effect in this model. On the other hand, codeine showed a marked inhibitory effect on the tail-flick response in rats. Codeine is a centrally acting agent that is administered orally and can be used for mild to moderate pain. The mechanism of action of diclofenac is achieved through an inhibition of COX enzyme activity, which results in a decreased production of PGs. These PGs are potent mediators of pain that act directly at nociceptors to increase nociceptor sensitivity (64) as well as act indirectly by enhancing pain-producing effect of other agents such as 5-HT or BKs (65). Hence, inhibition of PGs production results in analgesia. The results from acetic acid-induced writhing response and tail-flick test, therefore, suggest that the analgesic effect of AP extract is exerted peripherally. The mechanism of this effect is probably due to the blockade of the effect or the synthesis or the release of PGs and other pain mediators similar to diclofenac and other NSAIDs.

Inflammatory research involves various experimental models to study anti-inflammatory activity of test substance. These models are classified to be acute and chronic inflammatory models. Acute inflammatory model is designed to test drugs that affect vascular permeability, modulate leukocyte migration and chemotaxis (66). In topical acute inflammation model, mouse ear edema could be induced by several irritants such as EPP, AA, 12-0-tetradecanoylphorbol 13-acetate (TPA), capsaicin, zymosan, carrageenin, croton oil and mustard oil. EPP-induced ear edema is a useful model for screening and investigating the anti-inflammatory activity of test substances on acute phase of inflammation. This model is a rapid and uncomplicated test, requires small quantities of substances and provides well-reproducible results (67).

EPP application causes the release of various inflammatory mediators including histamine, 5-HT and PGs and thereby induces the vasodilatation, increases vascular permeability and produces edema (68). Moreover, the application of EPP has been reported to cause epidermal hyperplasia and inflammation (69). In the present study, AP extract and diclofenac produced significant inhibitory activity on edema formation evoked by EPP, therefore, the anti-inflammatory activity of AP extract may be due to the inhibition of the synthesis or the release of the inflammatory mediators found in the acute phase of inflammation.

Among several *in vivo* models of acute inflammation, the most frequently used model is the carrageenin-induced hind paw edema in rats or mice. The inflammation induced by carrageenin in the rat paw, originally described by Winter *et al.* (1962), is acute, non-immune, well-researched and highly reproducible (52, 67). This inflammatory model is commonly used for determining the anti-inflammatory activity of test compound of which its mechanism involves COX inhibition (52, 70). Injection of carrageenin, a mucopolysaccharide derived from *Chondrus crispus*, into the rat paw produces three phases of inflammation. The first phase, during the first 1.5 h, is mediated by histamine and 5-HT; the second phase (1.5 to 2.5 h) is mediated by BKs; and the third phase is attributed to local production of PGs from 2.5 to 6 h after carrageenin injection (71). Typically, test compounds are assessed for acute anti-inflammatory activity by examining their ability to reduce or prevent the development of carrageenin-induced paw swelling. In the present study, the edema volume of rat paw in the control group (received distilled water), was increased gradually and reached maximum increase at the 3rd to the 5th h after carrageenin injection. Various doses of AP extract and reference drug diclofenac exhibited significant edema inhibition at all recorded times. Moreover, the percent inhibition of AP extract at all doses on the edema formation was gradually increased as the dose increased. It is suggested that AP extract exhibits anti-inflammatory activity by an inhibition of the release and/or synthesis of various inflammatory mediators including histamine, 5-HT, BKs and especially PGs which are associated in inflammation and pain.

AA-induced paw edema in rat is a potentially useful model for detecting anti-inflammatory of LOX inhibitors and other agents with a mechanism of action different from COX inhibitors. AA has previously been demonstrated to produce a

small but significant inflammatory edema (72). Moreover, edema produced by AA is extremely sensitive to inhibition by dual inhibitors of AA metabolism (e.g., phenidone), corticosteroids (e.g., prednisolone) and LOX inhibitors (e.g., zileuton) but is insensitive to COX inhibitors (53). In this study, oral administration of AP extract and diclofenac did not significantly inhibit AA-induced edema. In contrast, the administration of prednisolone produced inhibition of AA-induced edema. These results indicate that anti-inflammatory activity of AP extract is not related to the LOX inhibition.

The cotton pellet-induced granuloma formation in rats is a typical model which established as a chronic inflammatory reaction (73). The inhibitory effect of AP extract on chronic stages of the inflammatory process was evaluated for its ability to reduce the deposition of granulation tissue around implanted cotton pellets. The response to subcutaneously implanted cotton pellet in rats has been divided into three phases, transudative, exudative and proliferative phases. The transudative phase is defined as the increase in the wet weight of the granuloma that occurred during the first three hours whereas the proliferative phase is defined as the increase of dry weight of the granuloma occurred between three and six days after implantation. The reduction of transudative weight of anti-inflammatory drugs involves in the inhibition of the permeability response of the blood vessels around the cotton pellet implantation whereas the inhibition of granuloma formation is probably via the interference with proliferative component such as fibroblasts, collagen and mucopolysaccharide during granuloma tissue formation (54, 74). In addition, Swingle and Shideman (1972) described that NSAIDs show a slight inhibition whereas steroids prove to be potent inhibitors on both the transudative and proliferative phases (54). In the present investigation, AP extract and diclofenac were significantly effective only in inhibiting the transudative whereas prednisolone exhibited strong inhibitory effect on both phases. The results obtained suggest that AP extract inhibits only the vascular permeability but not proliferative phase of chronic inflammation.

Steroids such as prednisolone can prevent or suppress inflammatory reactions. Chronic use of steroids induces the loss of body weight gain and thymus weight of rodents. These steroidal effects may be due to increase in protein catabolism and lymphoid tissue destruction (75). In the present study, AP extract and diclofenac did

not influence body weight gain and thymus weight of the rats whereas prednisolone elicited marked reduction of both parameters. Therefore, the anti-inflammatory activity of AP extract does not share the steroidal-like activity.

The migration of leukocytes to the injury site is occurred during chronic inflammation. Leukocytes accumulation leads to the release of lysosomal enzymes and oxygen radicals at inflammatory site (76). In cotton pellet-induced granuloma formation, the activity of lysosomal enzymes such as ALP in serum, is markedly elevated on the 7^{th} day after implantation (77) and normalized by NSAIDs and steroids through the stabilization of lysosomal membrane and inhibition of the migration of the inflammatory cells into inflammatory sites (76, 78). In the present study, only diclofenac and prednisolone but not AP extract could normalize ALP activity in rats. The results obtained suggest that AP extract cannot stabilize the lysosomal membrane or inhibit the migration of leukocytes during chronic inflammation.

Taken together, the mechanism of action of AP extract seems to be similar to that of diclofenac, except it did not normalize the elevated ALP activity as did diclofenac. Furthermore, AP extract did not produce the gastric ulcer. The anti-inflammatory activity but deprivation of ulcerogenic effect is a clinical desirable characteristic of novel anti-inflammatory agents, and AP extract fulfills this criterion.

Zingiberaceous plants are well-known for their use as medicinal herbs. *A. purpurata* consists of several phytochemical constituents such as 3-methoxyflavone, steroidal glycosides (32), unstable labdane diterpene, and alkaloid piperine (33). The pharmacologically active compounds diterpene and alkaloid have been shown to have anti-inflammatory activity (79). Moreover, the hydroalcoholic extract of *A. purpurata* consists of two flavonoids, rutin and kaempferol-3-O-β-D-glucuronide. It has previously been proved that plant flavonoids show potent antioxidants and anti-inflammatory effects (36). Therefore, it is suggested that the anti-inflammatory activity of AP extract may be due to diterpene, alkaloid and flavonoid constituents found in this plant.

The toxicity test in rats has long been used as a model for testing the safety of various agents. For investigation and assessment of the toxic effect of AP extract, the acute oral toxicity in rats was performed. According to OECD guideline (2001), the

lower limit of dose of test substance for acute oral toxicity testing is 2,000 or in certain circumstance 5,000 mg/kg. In this study, a single oral administration of AP extract at the dose of 2,000 mg/kg body weight did not produce any mortality, toxic signs, or other abnormal physiological activities when compared with those of the control group. Moreover, there were no visible abnormalities and no differences in size and color of the internal organs of rats in all groups. These results demonstrate its safety and lend support to its use to relief acute pain and inflammation. However, further studies such as subchronic and chronic toxicity tests of AP extract should to be conducted to confirm its safety.

4.2 Conclusion

The results of the present study suggest that AP extract possesses analgesic and anti-inflammatory effects. The analgesic activity of AP extract was evidenced by the significant reduction of acetic acid-induced writhing response in mice. It is likely that AP extract exerts its analgesic effect by inhibiting peripherally mediated nociception. The anti-inflammatory effect of AP extract was found only on acute inflammation. Acute anti-inflammatory reaction of AP extract was evidenced by the significant reduction of edema formation including EPP-induced ear edema and carrageenin-induced paw edema in rats. On the other hand, AP extract did not have inhibitory effect on AA-induced paw edema in rats. It seems that the anti-inflammatory effect of AP extract probably mediates via the inhibition of COX, but not of LOX pathway or inhibition of the synthesis and/or the release of other mediators, e.g., histamine, 5-HT and BKs. In the chronic inflammation study, AP extract reduced only the transudation but did not diminish the body weight gain and thymus weight in cotton pellet-induced granuloma formation in rats. Therefore, it seems that the anti-inflammatory activity of AP extract is devoid of steroidal-like activity. In addition, the oral administration of AP extract at a dose of 2,000 mg/kg did not produce mortality or any signs of toxicity or changes in general behavior and abnormality of the internal organs. These obtained results demonstrate the safety and lend support to the use of AP rhizome extract to relief acute pain and inflammation.