# CHAPTER 4

# DISCUSSION AND CONCLUSION

## Discussion

### Analgesic

The analgesic activity of TI extract was evaluated using both chemical (acetic acid-induced writhing response) and thermal (tail-flick test) methods. The acetic acid-induced writhing response in mice is commonly tested for detection of peripheral and central analgesic acting of drugs. Intraperitoneal injection of acetic acid induces tissue damage and causes the 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 terminal (peripheral sensitization), leading to phenotypic alteration of the sensory neurons and increased excitability of the spinal cord dorsal horn neurons (central sensitization) (76, 77). Consequences of peripheral sensitization are 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 (78). 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 (62). The results from the study showed that pre-treatment with diclofenac and various doses of TI extract significantly inhibited the writhing response caused by acetic acid injection in dose-dependent manner. The obtained data imply the analgesic activity of TI extract. To elucidate mechanism of analgesic activity involving CNS pain pathway, 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) reaction (79). The rat tail-flick test is a measure of acute cutaneous thermal pain and is generally considered to be a measure of nociceptive threshold (80). It is well-known that pain produced in the tail-flick test is sensitive and inhibited by centrally acting analgesic drugs such as morphine and codeine, which was also evidenced in the present study. The maximal dose of TI extract (200 mg/kg) used in the present study, and reference drug 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. It is a weak opioid analgesic with weak affinity to Mu (μ) opioid receptors which found primarily in the brainstem and medial thalamus. Opioid receptors are located on the presynaptic terminals of the nociceptive C-fibers and A delta-fibers. Once the receptors are activated, they will indirectly inhibit the voltage-dependent calcium channels, decrease the cAMP levels and block the release of pain neurotransmitters such as glutamate, substance P, and calcitonin gene-related peptide from the nociceptive fibers, resulting in analgesia (81). The mechanism of action of diclofenac to relieve nociceptive pain or inflammatory pain 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 (82) as well as act indirectly by enhancing pain-producing effect of other agents such as 5-HT or BKs (83). Hence, inhibition of PGs production results in analgesia. The results obtained from acetic acid-induced writhing response and tail-flick test therefore suggest that the analgesic effect of TI extract exerted peripherally pain pathway. The mechanism of this effect is probably due to the blockade of the peripheral effect of pain mediators.

**Inflammation**

Anti-inflammatory activity of test substance was studied by using various experimental models. These models are classified into acute and chronic inflammatory models.

Acute inflammatory model is designed to test drugs that affect vascular permeability, modulate leukocyte migration and chemotaxis (84). In topical acute inflammation model, ear edema is induced by several irritants such as EPP, AA, 12-0-

tetradecanoylphobol 13-acetate (TPA), capsaicin, zymosan, carrageenan, croton oil and mustard oil. EPP-induced ear edema is a useful model for screening and investigating the anti-inflammatory activity of test substances. This model is a rapid and simple test, requires small amount of substances and provides well-reproducible results (85). EPP application causes the release of various inflammatory mediators including histamine, 5-HT and PGs and thereby induces the vasodilation, increases vascular permeability and produces edema (86). Moreover, the application of EPP has been reported to cause epidermal hyperplasia and inflammation (87). In the present study, TI extract and diclofenac produced significant inhibitory activity on edema formation evoked by EPP, suggesting that TI extract possesses the anti-inflammatory effect. The mechanism of action of anti-inflammatory effect may involve the inhibition of the synthesis, release or action 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 carrageenan-induced hind paw edema in rats or mice. The inflammation induced by carrageenan in the rat paw, originally described by Winter *et al*. (1962), is acute, non-immune, well-researched and highly reproducible method (67, 88). This inflammatory model is commonly used for determining the anti-inflammatory activity of test compound of which its mechanism involves COX inhibition (67, 89). Injection of carrageenan, 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 (1.5-2.5 h) is mediated by BKs, and the third phase is attributed to local production of PGs from 2.5 to 6 h after carrageenan injection (90). Typically, test compounds are assessed for acute anti-inflammatory activity by examining their ability to reduce or prevent the development of carrageenan-induced paw swelling. In the present study, the edema volume of rat paw in the control group (received 5% Tween 80), was increased gradually and reached maximum increase at the 3$^{rd}$ h after carrageenan injection. TI extract at various doses and diclofenac exhibited a significant paw edema inhibition at all recorded times except TI extract at the dose of 50 mg/kg at the 5$^{th}$ h. The duration of action of the low dose of TI extract (50 mg/kg) was less than 5 h since it might be metabolized or excreted from the body before 5 h. Moreover, the percent inhibition of TI extract on the edema formation was gradually increased as the dose increased. It is suggested that TI extract exhibits anti-

inflammatory effect and the mechanism of action may partly involve COX pathway. Additionally, the mechanism of anti-inflammatory effect may involve an inhibition of the release and/or synthesis of various inflammatory mediators such as histamine, 5-HT, BKs and 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 with a mechanism of action different from COX inhibitors. AA has previously been demonstrated to produce significant inflammatory edema (91). Moreover, edema produced by AA is extremely sensitive to inhibition by corticosteroids (e.g., prednisolone, dexamethasone), dual inhibitor of AA metabolism (e.g., phenidone) and LOX inhibitor (e.g., zileuton) but is insensitive to COX inhibitors (68). In this study, oral administration of prednisolone and TI extract at the dose of 100 and 200 mg/kg significantly inhibited AA-induced paw edema. Moreover, the percent inhibition of TI extract at all doses on the edema formation was gradually increased in dependent manner. Taken together with the results from carrageenan-induced paw edema, it indicates that anti-inflammatory effect of TI extract may relate to inhibition of both COX and LOX pathway of AA metabolism.

The cotton pellet-induced granuloma formation in rats is a typical model which established as a chronic inflammatory reaction (92). The inhibitory effect of TI extract on chronic stages of the inflammatory process is 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, respectively. The transudative phase is defined as the increase in the wet weight of the granuloma. that occurred during the first 3 h whereas the proliferative phase is defined as the increase of dry weight of the granuloma occurred between 3-6 days after implantation. The reduction of transudative phase 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 (69, 93). In addition, Swingle and Shideman (1972) described that NSAIDs showed a slight inhibition whereas steroids prove to be potent inhibitors on both the transudative and proliferative phases (69). In the present investigation, TI extract did not inhibit the

transudative and granuloma formation when compared to those of the control group which received 5% Tween80. Prednisolone-treated group showed the superior inhibitory effects on both phases than those of diclofenac-treated group. The results obtained suggest that TI extract did not affect both transudative and proliferative phase of chronic inflammation.

Steroids such as prednisolone can prevent or suppress inflammatory reaction. 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 (94). In the present study, TI extract and diclofenac did not influence body weight gain and thymus weight of the rats whereas prednisolone-treated rats showed marked reduction of both parameters. Therefore, the anti-inflammatory activity of TI extract that showed in acute inflammation did 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 (95). In cotton pellet-induced granuloma formation model, the activity of lysosomal enzyme such as ALP in serum, is markedly elevated on the $7^{th}$ day after implantation (96) and can be normalize by NSAIDs and steroids through the stabilization of lysosomal membrane and inhibition of the migration of the inflammatory cells into inflammatory sites (95, 97). In the present study, the serum ALP activity of diclofenac- and prednisolone-treated groups was significantly decreased when compared with that of the control group. In TI extract-treated group, the serum ALP activity was not changed from that of the control group. The results obtained suggest that TI extract had no effect on chronic phases of inflammation.

The phytochemical screening test of TI extract demonstrated the present of alkaloids. Many plant-contained alkaloids such as cassiaindoline in *Cassia alata* and alkaloid fraction in *Ziziyphus nummularia* were proved to possess analgesic and anti-inflammatory activities (98, 99). Although, the exact compound responsible for analgesic and anti-inflammatory activities remains unclear, our data seem to relate to the traditional use of *Tacca integrifolia* in inflammatory conditions.

**Anti-ulcerogenic**

It is well known that the peptic ulcer develops when aggressive factors overcome defensive factors (100). It is now clear that NSAIDs induce injury account for the majority cause of peptic ulcer (101). Different therapeutic agents including plant extracts are used to treat peptic ulcer. The study on new gastroprotective agent commonly uses the different models with different mechanisms of ulcerogenesis. In the present study, TI extract showed anti-ulcer activity in experimental models, included EtOH/HCl- and indomethacin-induced gastric lesions.

TI extract and ranitidine, a $H_2$-receptor antagonist, significantly decreased gastric ulcer formation in the EtOH/HCl-induced gastric ulcer in rats. The effect of EtOH/HCl depends on both the concentration and the duration of exposure. The lesions are characterized by multiple-hemorrhage red bands of different sizes along the long axis of the glandular stomach (102, 103). The gastric lesion produced by EtOH is due to its direct necrotizing action causing a reduction of gastric mucosal defensive factors. EtOH causes disruption of the physiological function of the gastric mucosa throughout the glandular stomach in rats (104), resulting in reduction of the transmucosal potential difference. This phenomenon leads to an increase net flux of $Na^+$ and $K^+$ across the membranes into lumen, and an increased $H^+$ back-diffusion through the gastric mucosa (105). The diffusion of acid into the gastric mucosa is increased when HCl is used in combination with EtOH (106). Additionally, EtOH can cause acute gastric mucosal damage by disturbance of gastric microvascular circulation resulting an increase of microvascular permeability and followed by microvascular stasis (107-109). This event plays role in the development of EtOH-induced gastric mucosal damage. The microvascular stasis results in the failure of oxygen and nutrition delivery, as well as dilution and carry away back-diffusion of $H^+$ (108). The possible mechanisms of TI extract to reduce gastric lesions in this model could be the stimulation of mucus and/or bicarbonate secretion (110). The exact mechanism of action of TI extract was further investigated.

The most common adverse effect of the use of indomethacin is the development of gastric ulcer (111, 112). Indomethacin inhibits PG biosynthesis by inhibiting both COX-1 and COX-2 (106) resulting in over production of LTs. PGs, especially $PGI_2$ and

$PGE_2$, have a cytoprotective effect on gastric mucosa, which possibly mediated via increased mucosal blood flow (106), promotion of gastric mucus and bicarbonate secretion (113-115). $LTB_4$, an active metabolite from 5-LOXs, which plays roles in the production of proinflammatory cytokines, contributing the gastric damage (114) by stimulating of the polymorphonuclear leukocytes aggregation and adhesion of neutrophils to vascular endothelial cells causing gastric vasoconstriction and generation of reactive oxygen metabolite resulting in gastric ulcer (116). Because the anti-inflammatory effect of TI extract involved the inhibition of LOX pathway that proved in AA-induced paw edema experiment, the significant inhibitory effect of TI extract in indomethacin-induced gastric ulcer indicates that the gastroprotective effect of TI extract may involve the inhibition of LTs biosynthesis and/or LTs effects. Additionally, the decrease of gastric acidity or the induction of mucus may be involved (117-119).

The mechanism of stress-induced gastric ulceration suggests the involvement of sympathetic as well as parasympathetic nervous function which innervates the digestive organs (120, 121). Many endogenous substances are known to affect HCl secretion by the parietal cell including with ACh from the vagus nerve, histamine from mast cell, and gastrin from G cell. Moreover, stress-induced gastric ulcers can be prevented partly or entirely by vagotomy (122, 123), anti-cholinergic agents (124), and anti-secretory agents such as $H_2$-receptor antagonists and proton pump inhibitors (125, 126). TI extract did not show gastroprotective effect at all given doses on stress-induced gastric ulcer, but ranitidine significantly inhibited stress-induced gastric ulcer formation. Thus, the mechanism of TI extract to prevent gastric ulcer in EtOH/HCl and indomethacin experiments did not involve with anti-cholinergic and anti-secretory effects. TI extract was tested in the pylorus ligation model for investigation the possible mechanism of anti-secretory effect (73). The ligation of the pyloric end of the stomach causes stimulation of secretion and accumulation of gastric acid in the stomach which leading to auto-digestion of gastric mucosa and breaking down of the gastric mucosal barrier (127, 128). The results showed an anti-secretory activity of ranitidine at the dose of 100 mg/kg by significant decreases of both gastric volume and total acidity in ranitidine-treated group. TI extract, in the contrary, did not show any effect on both gastric volume and total acidity.

The effect of the TI extract on gastric wall mucus was then studied in another the EtOH/HCl-induced gastric ulcer model (74). EtOH/HCl is a necrotizing agent causes a decrease of gastric wall mucus after administration (106). The amount of gastric wall mucus was found to decrease in the control and ranitidine groups. Whereas those of the TI extract at the dose of 200 mg/kg and misoprostal ($PGE_1$ analogue) groups were higher than that of the control group. The results showed that TI extract possibly enhances the synthesis and/or secretion of the gastric mucus and may preserve the construction of gastric mucus interrupted by necrotinizing agent such as EtOH/HCl.

The finding obtained from the present study suggests that TI extract prevented gastric ulcer formation in EtOH/HCl- and indomethacin-induced gastric lesions and the possible mechanisms mediating the gastric ulcer protective activity may relate to a preservation and/or synthesis of the gastric mucus. However, TI extract did not show any effect in restraint water immersion stress-induced gastric lesions and pylorus ligation models, thus the mechanism of TI extract did not involve the inhibition of gastric acid secretion. In conclusion, TI extract exhibits gastroprotective effect via preservation and/or synthesis of the gastric mucus. Our results support the potential use of *T. integrifolia* in traditional medicine and may lead to verify and develop the major active component(s) to be used for gastric ulcer treatment and prevention in the future.

**Toxicity**

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 TI extract, the acute oral toxicity in rats was performed. According to OECD guideline (2001), the lower limit of test substance for oral toxicity testing is 2,000 mg/kg. In this study, a single oral administration of TI extract at the dose of 2,000 mg/kg body weight did not produced any mortality, toxic signs, or other abnormal physiological activities when compared to 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 no toxicity in acute toxicity test. However, further studies such as subchronic and chronic toxicity tests of TI extract should to be conducted to confirm its safety.

**Conclusion**

The ethyl acetate extract of *T. integrifolia* leaves possesses analgesic and anti-inflammatory effect on acute inflammation. The mechanism of analgesic effect may be due to the inhibition of peripherally mediated nociception and the mechanism of anti-inflammatory effect may be via the inhibition of COX and LOX pathways. TI extract does not share steroidal-like action or produce any toxicity in acute oral toxicity test in rats. Moreover, TI extract exhibits gastroprotective effect via preservation and/or synthesis of the gastric mucus. Our results suggest that *T. integrifolia* has potential to be used as traditional medicine and may lead to verify and develop the active compounds possess anti-inflammatory and anti-gastric ulcer activity in the future.