CHAPTER 4

Discussion and Conclusion

Murdannia loriformis (ML) is the plant in family Commelinaceae. In the present study, the ethanol extract of its whole plant was screened for its phytochemical components using the standard methods (Ayoola *et al*., 2008; Yadav and Agarwala., 2011). The results revealed that ML extract composes of alkaloids, phenolic and tannins, anthraquinone glycosides, flavonoids glycosides, cardiac glycosides, sterol glycosides or triterpene glycosides. ML extract was thus expected to possess antioxidant activity since these phytochemical agents such as phenolic compounds and flavonoids are a source of antioxidant agents (Perron and Brumaghim, 2009; Brewer, 2011). This study using the antioxidant screening by DDPH radical scavenging assay confirmed that ML extract exhibited antioxidant activity.

Antioxidants status plays an important role on the control of the inflammation process. The infiltration of phagocytic leukocytes, such as neutrophils, monocytes, macrophages and eosinophils, into the inflamed area leads to the large production of oxygen free radicals, which are highly active to chemical reactions with other molecules (Valko *et al*., 2007). The antioxidant compounds possibly delay oxidation process by inhibiting formation of free radicals or by interrupting propagation of the free radical by one (or more) of several mechanisms (Brewer, 2011). Therefore, the antioxidant supplementation may provide a useful approach in attenuating cell injury and dysfunction observed in inflammatory disorders (Conner and Grisham, 1996). Anti-inflammatory activity of medicinal plants were reported to be correlated well with its antioxidant activity (Ravipati *et al*., 2012). Thus, the anti-inflammatory activity of ML extract was explored in this study using both *in vitro* and *in vivo* experimental models.

Activation of macrophages by stimuli, such as the bacterial endotoxin, LPS, and viruses increases the production of numerous inflammatory mediators, including nitric oxide

(NO), prostaglandin E_2 (PGE_2), and various cytokines (Lee *et al*., 1992). The anti-inflammatory property of ML extract was evaluated *in vitro* by measuring its ability to inhibit the LPS-activated production of NO in RAW 264.7 cells. Gallic acid (GA) was used as a positive control in this experiment since many studies report its anti-inflammatory effect (Murase *et al*., 1999; Kim *et al*., 2006). The result showed inhibitory effect of ML extract on NO production at both 24 and 48 h. Although ML extract was unable to reduce NO production in the post-treatment experiment, nevertheless, cells pre-treated with ML extract showed a significant reduction of NO production as same as GA did. These results implied that pre-treatment with ML extract may trigger anti-inflammatory environment within RAW 264.7 cells. The treatment of ML extract and GA did not affect the cell viability indicating that the reduction of NO production caused by ML extract, or GA did not result from decreased number of these cells.

NO is one of the inflammatory mediators involved in the inflammatory process. ML extract was further studied the suppressive effect on inflammatory genes expression using RT-qPCR technique. The inflammatory genes tested are TNF-α, IL-1β, IL-6, iNOS, and COX-2. The results revealed that pre-treatment of RAW264.7 cell with ML extract (or GA) reduced the expression of these inflammatory genes. It has been reported that pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 involves in the pathogenesis of RA. These cytokines participate in inflammation and bone destruction phase in RA (Komatsu and Takayanagi, 2012). It is likely that ML extract could suppress the pathogenesis of RA via down regulating those inflammatory genes expression.

Previous study revealed that ML extract possesses anti-inflammatory activity on both acute and chronic inflammation. This extract reduced granuloma tissue formation in the rat implanted with cotton pellet without side effect on gastric mucosa (Somja, 2005). As the cotton pellet-induced granuloma formation model represents chronic inflammatory diseases, this extract may be effective in chronic arthritis in the rats as well. Therefore, it is reasonable to determine whether ML extract exerts anti-arthritic activity in the animal model.

Induction of arthritis by complete Freund's adjuvant (CFA) is one of the standard methods used for investigation of anti-inflammatory and anti-arthritic agents. CFA-induced arthritis shares many features with human RA (Goodson *et al*., 2003; Durai *et al*., 2004). The injection of Freund's adjuvant, containing inert *Mycobacterium tuberculosis*, causes development of arthritis in rats during 10-45 days after injection (Pearson and Wood, 1959). The pathological features of adjuvant arthritis are polyarticular inflammation and edema (Bendele *et al*., 1999).

In the present study, an oral administration of ML extract in arthritic rats caused the decrease in paw edema volume at both sides similarly to that found in the indomethacin-treated group. Indomethacin could reduce paw edema in arthritic rats by inhibiting PG formation through inhibition of both COX-1 and COX-2 enzymes. During the inflammation process, local up-regulation of COX-2 biosynthesis leads to the increase of PG production. Overproduction of PGs exacerbates inflammatory response (Needleman *et al*., 1986; Salvemini *et al*., 1993). PGE_2 has been reported to involve in acute and chronic inflammatory processes (Ricciotti and FitzGerald, 2011; Turull and Queralt, 2000; Claveau *et al*., 2003). Both COX-2 and microsomal PGE synthase 1 (mPGES-1) also caused the PGE_2 production. These enzymes are highly expressed in the synovium of patients with RA and in models of arthritis (Sano *et al*., 1992; Sheibanie *et al*., 2007).

The *in vitro* studies revealed that rheumatoid synovial cells produce high levels of PGE_2 that can accelerate bone resorption by osteoclasts (Dayer *et al*., 1976; Bingham, 2002; Inada *et al*., 2006). Moreover, the levels of PGE_2 and COX-2 protein in paw tissue correlate well with an increasing of paw edema volume (Anderson *et al*., 1996). Therefore, anti-arthritic mechanism of ML extract may be partly through the inhibition of PGE_2 and/or inhibition of NO production.

The large amounts of inflammatory mediators such as NO and PGE_2, are generated by iNOS and COX-2 during the inflammatory process (Lee *et al*., 1992). It has been reported that NO plays an important role in the pathogenesis of joint inflammation and tissue damage in RA (Connor *et al*., 1995). Farrell and co-workers found an increasing of nitrite in the serum and synovial fluids of RA and OA patients when compared with healthy control (Farrell *et al*., 1992). The expression of iNOS was found in the synovial

lining layer, subsynovium, vascular smooth muscle and chondrocytes of the RA patients (Mazzetti *et al*., 2001). NO produced by iNOS may contribute to the pathogenesis of inflammatory arthritis by increasing the synovial blood flow, modulating cellular function within the synovium and articular cartilage (McCartney-Francis *et al*., 1993) and maintenance of the swelling in arthritis rats (Oyanagui, 1994). It has been noted that NO enhances COX activity resulting in an increasing of PGs production at the inflammatory site.

The basic pathological feature of RA is the infiltration of leukocytes into the inflamed joint, the proliferation of synovial tissue and angiogenesis (Bendele *et al*., 1999). The results from histological examination showed the infiltration of inflammatory cells and synovial membrane proliferation in the ankle joint of the control arthritic rats. In the indomethacin-treated group, the joint tissue showed the reduction of both infiltrated cells and proliferated synovial membrane. Indomethacin has been reported to decrease the cellular infiltrate in affected joints and inflammation of synovial tissue by reducing COX-2 and IL-6 protein levels in paw tissue (Anderson *et al*., 1996).

The cytokines TNF-α, IL-1β, IL-2, IL-6, and IL-23, play important roles in the inflammation and bone destruction in RA (Brennan and McInnes, 2008). TNF-α stimulates the release of chemokines that attract leukocytes from the blood into the inflamed tissue. It causes up-regulation of adhesion molecules and induces other proinflammatory cytokines production (IL-1 and IL-6) (Brennan and McInnes, 2008). IL-1β and TNF-α have an overlapping effect on pathogenesis of RA. They stimulate the production of inflammatory mediators such as PGE_2 and NO, IL-6, as well as increase adhesion molecules, chemokines, collagenases and COX-2 (Dinarello CA, 2001; Kwan Tat *et al*., 2004).

In adjuvant-induced arthritis, endogenous PGE_2 stimulates cytokine production, mainly IL-6. IL-6 involves in synovial cell proliferation in the synovium of RA patients (Mihara *et al*., 1995). Besides, this cytokine stimulates pannus development (Dayer and Choy, 2010), and promotes osteoclastogenesis and joint destruction (Mori *et al*., 2011).

Similar to indomethacin, ML extract at the dose of 400 mg/kg reduced the infiltration of inflammatory cells and decreased synovial membrane proliferation in the ankle joint

tissue of the arthritic rats. This observation may result from an inhibitory effect of ML extract on the activity, the synthesis or the release of COX-2, IL-6 and TNF-α. ML extract decreased cell migration and fibroblast proliferation, therefore, its mechanism may be due to inhibiting these inflammatory mediators.

In summary, all of the results from *in vitro* and *in vivo* experimental models revealed that, ML extract has a potential to use for arthritis. Its anti-arthritic mechanisms may be due to the reduction of inflammatory genes TNF-α, IL-1β, IL-6, iNOS and COX-2 as well as NO and PGE_2.

The role of reactive oxygen species (ROS) has been discussed recently in immunopathogenesis of RA. Patients with inflammatory arthritis are reported to have an increasing of ROS in the synovial tissue (Mirshafiey and Mohsenzadegan, 2008). Since TNF-α is a key cytokine in RA, overproduction of TNF-α likely contributes to ROS releasing in patients with RA (Mirshafiey and Mohsenzadegan, 2008). Antioxidant agents might be useful for RA treatment. This concept has been supported by a study in the year 2000 that the radical scavenger 'tempol' reduces the degree of chronic inflammation and tissue damage in collagen-induced arthritic rats (Cuzzocrea *et al*., 2000).

Anti-arthritic activity has been previously found in several plant-derived phytochemicals such as flavonoids, triterpenes and polyphenols (Nanjundaiah *et al*. 2013). Total flavonoids from orange decreased the paw thickness and improved the pathological impairment of the ankle joint of adjuvant-induced arthritic rats. The mechanism might be due to inhibition of the inflammatory mediator production (Chen *et al*., 2010). In addition, lupeol, a pentacyclic triterpene from *Calotropis gigantean*, has been shown to possess anti-arthritic activity in adjuvant-induced arthritic rats. This compound reduced the paw swelling and the production of proinflammatory cytokines such as TNF-*α*, IL-1β, and IL-6 (Saratha and Subramanian, 2012). The phytochemical screening revealed that ML extract contained phenolic compounds, flavonoids and triterpenes. The anti-arthritic activity of ML extract may be due to the effect of these compounds.

The drugs for RA treatment are commonly divided into four groups: DMARDs, biologic DMARDs, steroids, and NSAIDs. However, these drugs have a limitation of use as they can produce many adverse effects, especially GI side effects (Jones, 2001). From this point, searching for new anti-inflammatory agents with gastroprotective effect and/or without GI side effect is still needed. From the preliminary investigation of gastroprotective activity, ML extract reduced gastric ulcer lesion induced by ethanol/hydrochloric acid. Therefore, it was interesting to investigate gastroprotective effect of ML extract in rats.

The pathogenesis of gastric ulcer results from an imbalance of aggressive gastric luminal factor and defensive mucosal barrier function (Malfertheiner *et al*., 2009). The aggressive factors are gastric juice acid, pepsin, bile reflux, NSAIDs, *H. pylori* bacteria and alcohol. The defensive factors are mucosal blood flow, surface epithelial cells, prostaglandins, phospholipids or surfactant, mucus, HCO_3^- secretion, gastric motility, mucosa impermeability against H^+ ion, heat shock protein, and others (Syam *et al*., 2009). PGs play a pivotal role in maintaining mucosal integrity (Robert *et al*., 1979). This protective effect of PGs is the result of several actions, including their abilities to inhibit acid secretion, stimulate both HCO_3^- and mucus secretion, increase mucosal blood flow, and modify the local inflammatory response induced by acid (Sung, 2010).

In the present study, firstly, ML extract significantly decreased ulcer formation induced by EtOH/HCl. The mechanism of its anti-ulcerogenic activity is probably due to the increase in mucosal resistance such as gastric blood flow, stimulation of PGs production or inhibition of 5-lipoxygenase pathway.

Secondly, ML extract inhibited gastric lesion induced by indomethacin. Indomethacin induces gastric ulcer by inhibiting COX resulting in the decrease of cytoprotective system and PG production (Matsui *et al*, 2011). It is likely that anti-gastric ulcer activity of ML extract may be due to an increased production of endogenous PGs and reduction of other aggressive factors. However, this explanation needs to be further explored.

Thirdly, ML extract inhibited gastric ulcer formation in the stress-induced ulcer model. The pathogenic mechanisms of stress-induced gastric mucosal lesions involve the

disturbance of gastric mucosal microcirculation (Kitagawa *et al*., 1979; Hemmer *et al*., 1980; Murakami *et al*., 1985), an increase of gastric secretion (Brodie *et al*., 1962; Kitagawa *et al*., 1979; Murakami *et al*., 1985) and an abnormal gastric motility (Watanabe, 1966). Thus, it is possible that the ML extract exerted anti-gastric effect by inhibiting gastric acid secretion and/or promoting PG production.

Gastric wall mucus plays a major role as a defensive factor against gastrointestinal damage. ML extract increased the amount of gastric wall mucus in the rats whose gastric ulcer induced by EtOH/HCl. This result suggests that ML extract exerted gastroprotective effect by increasing defensive factor similarly to a reference drug, misoprostol, a PGE_1 analogue. Finally, ML extract significantly reduced gastric acid secretion in the pylorus ligation model. The anti-secretory effect of ML extract therefore may lead to the reduction of gastric ulcer in other ulcerogenic models.

Oxygen-derived free radicals has been reported to contribute to gastric mucosal damage in rats in which the gastric ulcer was induced by EtOH/HCl (Kanter *et al*., 2005), indomethacin (Yoshikawa *et al*., 1993), and stress (Das *et al*., 1997; Kwiecien et al., 2002; Li and Zhang, 1993). Free radicals also involve in the elevation of acid and pepsin contents of the gastric juice of pylorus ligated rats (Rastogi *et al*., 1998). Several reports showed that antioxidant may prevent gastric mucosa damage from free radicals (Alberghina *et al*., 1992; Ito *et al*., 1992; El-Missiry *et al*., 2001; Blandizzi *et al*., 2005; Kanter *et al*., 2005).

Many studies showed that alkaloids, flavonoids, tannins and triterpenes possess a gastroprotective activity in animal models (Aguwa, 1985; Ezaki *et al*., 1985; de Sousa Falcao *et al*., 2008; Zanatta *et al*., 2009; Souza *et al*., 2011). Flavonoids exerted anti-secretory, cytoprotective, antioxidant, and healing effects (Martin *et al*., 1988; Yamahara *et al*., 1990; Alarcon de la Lastra *et al*., 1994; Izzo et al., 1994; Arun and Asha, 2008; Mota *et al*., 2009) as well as increased mucosal PG content in rats (Alcaraz and Hoult, 1985). Therefore, flavonoids, alkaloids, tannins, and triterpenes found in ML extract may be responsible for its gastroprotective activity.

In the antioxidant activity screening, ML extract exhibited scavenging activity on the DPPH radicals. Since ROS contributes to gastric ulcer formation, anti-gastric ulcer activity of ML extract may be in part through its antioxidant activity.

Taken together, the mechanisms of anti-gastric ulcer activity of ML extract may mediate through its antioxidant activity as well as the reduction of aggressive factor, i.e. gastric acid and increasing defensive factor, i.e. mucus. ML extract was a crude extract and contained many phytochemical agents. In the present study, the search for active compound that exerts gastroprotective and anti-arthritic activities was not included. Further phytochemical study of ML extract is warranted to identify the active compounds.

Conclusion

The ethanol extract of *M. loriformis* (ML extract) has anti-arthritic activity as oral administration of this extract could reduce paw edema in arthritis rats. The histopathological study showed the reduction of both inflammatory cell infiltration and synovial hyperplasia in the ankle joint tissue. The mechanism of the extract may be due to the reduction of inflammatory gene expression including TNF-α, IL-1β, IL-6, iNOS, and COX-2. Moreover, ML extract showed gastroprotective effect in numerous models including the HCl/EtOH-, indomethacin- and restraint water immersion stress-induced gastric ulcer. The mechanisms of its anti-gastric ulcer activity may be due to the decreased gastric acid secretion and the increased gastric mucosal defense. As ML extract elicit anti-arthritic and gastroprotective effects, ML extract may be potentially used as an alternative agent for arthritis and/or peptic ulcer.