DISCUSSION

Inflammation is generally defined as the response of living tissue to an injurious stimulus, and is characterized by 5 symptoms i.e. redness, heat, pain, swelling and decreased function (Ammon *et al.*, 1993; Dunstan *et al.*, 1997). It involves a complex array of enzyme activation, mediator release, extravasation of fluid, cell migration, tissue breakdown and repair (Vane and Bolting, 1995). It is a complex process in which many different mediators are involved. Eicosanoids (i.e. prostaglandins, leukotrienes), platelet activating factor etc., have been reported to be involved in the development of inflammatory diseases (Campbell, 1990; Yesilada *et al.*, 1997).

Eicosanoids are synthesized from arachidonic acid by two families of enzymes, cyclooxygenase (COX) and lipoxygenase (LOX). These enzymes are capable of inserting oxygen into the molecule of arachidonic acid in a specific manner, thereby producing prostaglandins (PGs), by an action of COX, or leukotrienes (LTs), by an action of 5-LOX. The key enzyme in the synthesis of PGs from arachidonic acid, COX, was purified in 1976. After cloning of COX-1 gene in 1988, several laboratories identified a second gene with COX activity (COX-2) in 1991. The constitutive isoform of COX, COX-1, appears to posses a physiological function that involves the synthesis of cytoprotective PGs. These PGs may protect the stomach lining and maintain normal renal function in the kidney. The inducible isoform, COX-2, is induced by pro-inflammatory stimuli in migratory cells and in inflammed tissues. Because COX-2 is capable of regulating both pro-inflammatory cytokines and growth factors, this implies that it may play a role in inflammation and in the control of cell growth. *In vivo*, the primary role of PGs may be the regulation of cytokines and maintenance of the inflammatory cascade (Sugava *et al.*, 2000).

5-LOX is the enzyme responsible for the key step in leukotriene synthesis. The major inflammatory mediator produced by 5-LOX is leukotriene B_4 or LTB_4 (Sugava *et al.*, 2000). It is known to be a major product of activated neutrophils and macrophages

(Crooks and Stockley, 1998). In addition, complement activation can further propagate the inflammatory process by releasing the LTB_4 and PGE_2 (Barnum, 1995; Fiebich *et al.*, 1998).

Because most of the commonly used anti-inflammatory NSAIDs are basically nonselective COX inhibitors (i.e., effective in inhibiting both COX-1 and COX-2), the COX inflammatory pathway is currently being considered as an important mechanism. The non-selective COX inhibitors, i.e. NSAIDs, reduce the overall synthesis of PGs, including the PGs involved in protection of the gastric mucosa. Therefore, they are known to trigger gastric and duodenal ulcers. Although many patients obtain benefit with nonspecific COX inhibitor treatment, they also suffer from severe side effects such as gastrointestinal lesion and bleeding. The two COX isoenzymes share structural and enzymatic similarities but are differently regulated at the molecular level and may have distinct functions, although some physiological overlap between them does occur. In contrast, the selective COX-2 inhibitors are currently being developed with the expectation that these compounds will selectively suppress the prostanoid biosynthesis triggered by pathological events and that they will be devoid of the side effects associated with the inhibition of the constitutive prostanoid biosynthesis (Sugava *et al.*, 2000). 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 (Singh *et al.*, 1989).

In Thai folk medicine *V. harmandiana* is used for treatment of diabetic disease, chronic inflammation and wound (Pongboonroud, 1971). The claimed effect, i.e. anti-inflammatory activity, of *V. harmandiana* was first studied by Phankummoon (1998). Four methanolic extracts from this plant (from heart wood, bark and twigs) seemed to possess strong activity on the acute phase of inflammation but elicited weak inhibitory activity on the chronic phase of inflammation. PNQ-4482 is the major compound isolated from the heart wood of *V. harmandiana*. The previous preliminary screening using carrageenin-induced paw edema in rats revealed the anti-inflammatory activity of PNQ-4482. The preliminary toxicity testing showed that an oral single dose of 5,000

mg/kg of PNQ-4482 did not produce any signs or symptoms of toxicity in rats. Furthermore, PNQ-4482 at the dose of 300 mg/kg, given orally for 7 days, did not possess any ulcerogenic activity when compared with aspirin. In this present work PNQ-4482 was selected for the detailed study of its anti-inflammatory properties as well as its analgesic, antipyretic and anti-ulcerogenic activities. A Hippocratic screen and acute and subacute toxicity tests were included.

The acute inflammatory reaction is implicated as a host defense mechanism at the site of the microcirculation that comprises exudative and cellular responses. These responses are manifested by increased vascular permeability and leukocyte infiltration (Kikuchi *et al.*, 1991). The exudative response is relatively easy to study on the paw or ear edema formation technique (DiMartino *et al.*, 1987; Young *et al.*, 1984). It is well established that PGs, COX products of arachidonic acid (AA), are involved in inflammatory reactions as important inflammatory mediators, and inhibitors of PG biosynthesis have been developed as non-steroidal anti-inflammatory drugs (Ishii *et al.*, 1994).

EPP-induced ear edema formation is a useful model to investigate the anti-inflammatory activity of a test substance on the acute phase of inflammation (Brattsand *et al.*, 1982). The inflammatory mediators released in this model include histamine, serotonin, bradykinin and PGs. These mediators are capable of promoting vasodilatation and increasing vascular permeability as well as synergistically producing edema (Carlson *et al.*, 1985). The results of the present study show that PNQ-4482 exerted a dose-dependent inhibition of ear edema formation induced by EPP. Phenylbutazone, a selective cyclooxygenase inhibitor, could markedly reduce the edema of the ear in this model. It is suggested that PNQ4482 probably possessed anti-inflammatory activity, like phenylbutazone, by inhibition of the cyclooxygenase pathway and of other inflammatory mediators of the acute phase of inflammation.

Recently, it has been shown that LTs, 5-LOX products of AA, are also involved in inflammatory reactions as proinflammatory mediators. LTC_4 and LTD_4 cause edema together with increased microvascular permeability (Camp *et al.*, 1983; Peck *et al.*,

1981), and LTB_4 causes leukocyte chemotaxis (Palmblad *et al.*, 1981; Czarnetzki *et al.*, 1985). Experimental models of inflammation such as AA-induced ear edema have been widely used for the discovery and evaluation of anti-inflammatory drugs. AA produces an intense inflammatory reaction in the mice ear and subsequent experiments demonstrated that this response can be ameliorated by putative 5-LOX inhibitors. Thus, it would appear that lipoxygenase products are involved in this model of inflammation and serve as a suitable model for detecting 5-LOX inhibitors in *vivo* (Yong *et al.*, 1984; Chang *et al.*, 1986; Crummey *et al.*, 1987; Griswold *et al.*, 1987; Inoue *et al.*, 1988).

Selective cyclooxygenase inhibitors such as aspirin and phenylbutazone, produce no significant inhibition or are inactive in this model, whereas phenidone, a dual inhibitor of arachidonic acid metabolism shows consistently significant inhibition in this ear edema model (Yong *et al.*, 1984).

The present study showed that a topical application of AA to mouse ear caused ear edema. At this dose of AA, LTs are fully operating in inducing edema (Ishii *et al.*, 1994). Phenidone, a nonselective inhibitor of arachidonic acid metabolism, exhibited pronounced edema inhibitory effect on arachidonic acid-induced ear edema formation whereas phenylbutazone, a selective cyclooxygenase inhibitor, produced no inhibitory activity on the edema formation. PNQ-4482 showed significant inhibitory effect on ear edema formation induced by arachidonic acid. These results rather indicate that the anti-edematous effect of PNQ-4482 is mediated through the inhibition of LTs biosynthesis. It is therefore suggested that PNQ-4482 exhibits anti-inflammatory activity in part by inhibition of the lipoxygenase pathway.

Carrageenin-induced rat hind paw edema is considered as an acute inflammatory process which is well suited for the comparative bioassay of anti-inflammatory agents, since the relative potency estimates obtained from most drugs tend to reflect clinical experience (Winter *et al.*, 1962). The edema induced in the rat hind paw by the local injection of carrageenin is mediated (like many other edema processes) by the initial release of histamine and 5-HT and is followed by the release of

bradykinin during the 1st h after carrageenin injection, causing increased vascular permeability (Crunkhorn and Meacock, 1971; Di Rosa, 1972; Flower *et al.*, 1985). The second phase of inflammation is due to the release of PGs. PGs play a major role in the development of the second phase of reaction which is measured around 3 h after carrageenin injection and lasts about 7 h (Crunkhorn and Meacock, 1971; Di Rosa, 1972). The release of PGs is closely associated with leukocyte migration to the inflamed area (Di Rosa and Willoughby, 1971). The presence of PGE_2 in the inflammatory exudates from the injected foot can be demonstrated at 3 h and periods thereafter (Vinegar *et al.*, 1969). The carrageenin-induced rat hind paw edema model in rats is known to be sensitive to cyclooxygenase inhibitors and has been used to evaluate the effect of non-steroidal anti-inflammatory agents which primarily inhibit the cyclooxygenase involved in PGs synthesis. It has been demonstrated that the suppression of carrageenin-induced rat hind paw edema after the third hour correlates reasonably well with the therapeutic doses of most clinically effective anti-inflammatory agents (Di Rosa and Willoughby, 1971).

The significant inhibitory effect of PNQ-4482 on carrageenin-induced paw edema at the 3rd h, suggests that the main mechanism of action of PNQ-4482 may involve the PG-biosynthesis and/or release. PNQ-4482 may also possess some influence on the other mediators e.g. histamine and 5-HT which are released during the 1st h after carrageenin injection, since it showed pronounced inhibitory activity on the edema formation during this hour as well. The results in this test model support the possible mechanism of action of PNQ-4482 on the cyclooxygenase pathway and on other inflammatory mediators, which are involved in paw edema caused by carrageenin.

Carrageenin induced pleurisy in rat can serve as a more useful model for the analysis of the acute inflammation (Di Rosa *et al.*, 1971). The fluid extravasation, leukocyte migration and the various biochemical parameters involved in the inflammatory response can readily be measured (Capasso *et al.*, 1975).

In previous work from Imai *et al.*, (1991), Kikuchi *et al.*, (1994) and Murakami *et al.*, (1999) it was demonstrated that histamine, serotonin, platelet-activating factor (PAF), complement, and PGs were mediators causing exudation.

The complement system is activated at the early stage of rat pleurisy and the irritant injected into the pleural cavity activates the complement system by contact with the interstitial fluid. This activated complement thus enhances PAF synthesis, and possibly that of the other mediators, in the resident leukocytes. Since the exudate was reduced by only about half when complement and PAF were nullified, these components are not the only ones involved in exudate formation (Imai *et al.*, 1991). The plasma exudation involves two types of mediators: some are predominantly vasodilators (PGs) and some are important for their vascular permeability increasing activity (kinins, histamine and serotonin) (Williams, 1979). PGs, histamine and/or serotonin, and PAF could be responsible for induction of exudation, since antagonism of these mediators significantly suppressed the exudation process (Imai *et al.*, 1991; Murakami *et al.*, 1999).

The present study demonstrates that the steroidal anti-inflammatory drug (prednisolone), NSAIDs (aspirin) and PNQ-4482 elicited significant reduction of the pleural exudate formation at the 3rd h after carrageenin injection. Flower and Dale (1989) reported that the steroidal anti-inflammatory drugs such as prednisolone induce the synthesis of a protein inhibitor of phospholipase A_2, namely lipocortin, and result in inhibition of prostaglandin biosynthesis. Lo *et al.* (1981) indicated that NSAIDs could prevent PGs from potentiating the permeability-increasing mediators. Mikami and Miyasaka (1983) postulated that the anti-exudative effect of NSAIDs might be mainly due to the reduction of PGE_2 content. The reduction of pleural exudate formation produced by PNQ-4482 might also be due to the reduction of PGs and thereby decreasing vascular permeability, resulting in a decrease of exudate volume.

For studying leukocyte infiltration there are no suitable experimental systems except for the techniques using a body cavity (i.e., pleural or peritoneal cavity) or artificial body space (i.e., air pouch). Since these systems are not disease models, one

of the purposes of the investigation was to establish a simpler and more quantitative method to measure leukocyte infiltration than by histological examination (Kikuchi *et al.*, 1991). It has been reported that various cytotoxic inflammatory mediators are produced and released by leukocytes infiltrating into the inflamed tissue, causing the later phases of inflammation. Therefore, regulation of the leukocyte infiltration is important in the treatment of inflammatory disease, and can be an attractive target for the development of new anti-inflammatory agents. Many reports have shown that leukocyte infiltration is induced by various chemotactic mediators such as complement fragment (C5a), LTB_4or PAF (Capasso *et al.*, 1975; Sedgwick *et al.*, 1985; Kikuchi *et al.*, 1991). Carrageenin has been shown to activate the complement system through the alternative pathway (Roch-Arveiller *et al.*, 1977). Lo *et al.* (1984) and Movat *et al.* (1984) reported that the major component of the exudate chemotactic activity is thought to be protein(s) related to complement component 5a (C5a), a highly chemotactic factor for polymorphonuclear leukocytes. More than 90% of the leukocyte accumulation elicited by carrageenin are constituted by neutrophils (Vannier *et al.*, 1989). It has also been reported that anti-inflammatory steroids inhibit the leukocyte infiltration by inhibiting production of chemotactic mediators (Kurihara *et al.*, 1984) and leukocyte motility (Rivkin *et al.*, 1976). In addition, Vinegar *et al.* (1976) found that inhibition of the migration of the neutrophils during carrageenin pleurisy is the principal action of the corticosteroids. Furst and Munster (2001) reported that aspirin, in addition to its inhibitory effect on COX, also inhibits the migration of polymorphonuclear leukocytes and macrophages into the site of inflammation.

The results of the present study showed that aspirin and prednisolone exhibited significant inhibitory effect on leukocyte accumulation in the pleural exudate. It was found that the inhibition of fluid extravasation by these drugs is well correlated with that of leukocyte accumulation. PNQ-4482 exhibited a significant inhibitory effect on leukocyte accumulation. From this result it may be postulated that PNQ-4482 inhibits leukocyte migration, like steroid or nonsteriodal agents, by reducing the extravasation of

plasma proteins, generation of chemotactic factors related to complement components and leukocyte chemotactic factors.

PGs are a family of biologically active lipids, which are present in many mammalian tissues, fluids and may be released after nerve or hormonal stimulation (Karim *et al.*, 1967; Karim and Hillier, 1968; Katori *et al.*, 1978; Pickles *et al.*, 1967). The amounts present in the tissue or released from them are so small that the available chemical assay methods are not suitable. The method generally used for quantitative determination of tissue prostaglandins involves a suitable solvent extraction, separation by thin-layer chromatography on silica gel, elution and bioassay on isolated smooth muscles against pure standard prostaglandin. The common tissue prostaglandins (PGE_1, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$) are powerful spasmogens ("slow-reacting substances") on many isolated smooth muscles. The commonest assay organs are rabbit duodenum, guinea pig ileum, hamster and gerbil colon, rat uterus and rat stomach fundus. Parallel bioassay of unknown prostaglandins on tissues with different sensitivities have been used to provide presumptive evidence of the type of prostaglandin present.

Both aspirin and prednisolone, standard drugs used in this study model, have been known as PG-biosynthesis inhibitors. Aspirin inhibits COX activity whereas prednisolone acts on phospholipase A_2 activity and thereby reducing the amount of AA and hence PGs synthesis.

Evaluation of PGE_2-like activity by bioassay on rat fundus strip showed that PNQ-4482, similarly to ASA and prednisolone, reduced the production of PGs, reflecting its interfering effect on the synthesis and/or release of these mediators.

The inflammatory granuloma is a typical feature of established chronic inflammatory reaction and can serve for investigation of anti-arthritic substances (Spector and Willoughby, 1959; Spector, 1969). The cotton pellet granuloma method has been widely employed to assess the transudative and proliferative component of

chronic inflammation. 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. Three phases of the response to a subcutaneously implanted cotton pellet have been described. These consist of 1) a transudative phase, defined as the increase in wet weight of the pellet which occurred during the first three hours, 2) an exudative phase, defined as leakage of plasma from the bloodstream around the granuloma and occurring between 3 and 72 h after implanting the pellet and 3) a proliferative phase, measured as the increase in dry weight of the granuloma which occurs between three and six days after implantation. Although the anti-inflammatory drugs can inhibit both the transudative phase and the proliferative phase, non steroidal anti-inflammatory agents give only slight inhibition whereas steroidal anti-inflammatory agents have a strong inhibition on both phases (Swingle and Shideman, 1972).

The effect of PNQ-4482 on the transudative and the proliferative phase of chronic inflammation was determined. The result showed that PNQ-4482 elicited significant inhibitory activity on the transudative and granulomatous weight. In addition, aspirin, a non-steroidal anti-inflammatory drug, and prednisolone, a steroidal drug, exerted marked inhibitory activity on the formation of transudate and granuloma. When assessment was made on the body weight gain and the thymus weight, it was found that only prednisolone markedly reduced the body weight gain and the thymus weight. Although steroids, particularly corticosteroids such as prednisolone, stimulate protein synthesis in liver, they have pronounced catabolic effects on lymphoid and connective tissue, muscle, fat and skin. The results obtained suggest a difference in mechanism of anti-inflammatory action of PNQ-4482 and prednisolone since PNQ-4482 did not influence the body and the thymus weight. It is therefore postulated that the mechanisms of anti-inflammatory action of PNQ-4482 are more similar to NSAIDs than to steroids.

Arachidonic acid metabolites, particularly LTB_4, can mediate or modulate leukocyte influx into inflammatory sites. LTB_4 is a potent chemotactic agent for polymorphonuclear leukocytes, eosinophils, and monocytes (Campbell and Halushka,

1996). Leukocytes accumulate at sites of inflammation and are believed to contribute to tissue damage by releasing lysosomal enzymes and toxic oxygen radical (Salmon and Higgs, 1987). The role of lysosomal enzymes such as alkaline phosphatase as mediators of inflammation is well documented (Weismmann, 1967; Becker and Henson, 1973). The activity of alkaline phosphatase raised in serum during the inflammatory process, results in the damage of tissue and cartilage that can lead to further perpetuation of the inflammation. The alkaline phosphatase is elevated during cotton pellet granuloma formation peaks on the seventh day and decreases by day 14 when healing occurrs (Bessey *et al.*, 1946; Nishikaze *et al.*, 1980). The lysosomal enzyme activity in serum and in the exudate elevated during inflammation can be normalised by both NSAIDs and steroidal drugs via the stabilization of lysosomal membrane (Salmon and Higgs, 1987). The activity of serum alkaline phosphatase raised in rats in cotton pellet-induced granuloma model in this present study was normalised by all test drugs i.e. prednisolone, aspirin and PNQ-4482. This result suggests the efficacy of PNQ-4482 and reference drugs in stabilizing the lysosomal membrane during chronic inflammation. Beside this, PNQ-4482 was proven in the arachidonic acid-induced ear edema model to inhibit the lipoxygenase pathway in producing LTs. This effect, especially inhibition of LTB_4 biosynthesis, can reduce leukocyte migration into the inflammatory area and phagocytic activity, thereby reducing lysosomal enzyme leakage from those cells.

Since the anti-inflammatory, analgesic and anti-pyretic properties of drugs in the nonsteriodal group are due to their inhibition on PGs-biosynthesis (Flower, 1985), the analgesic activity of PNQ-4482 was then evaluated using the writh response and the formalin test in mice, while the antipyretic effect was measured by yeast-induced hyperthermia in rats.

Acetic acid-induced writhing response in mice is widely used for analgesic screening (Alexandre-Moreira *et al.*, 1999). This test is normally used to study the peripheral and central analgesic effect of drugs. The abdominal constriction response

is thought to involve, in part, local peritoneal receptors (Eddy and Leimbac, 1953; Koster *et al.*, 1959; Bentley *et al.*, 1983; Alexandre-Moreira *et al.*, 1999). Acetic acid caused algesia by liberating endogenous substances including serotonin, histamine, PGs, bradykinin, substance P and many others that excite pain nerve ending (Raj, 1996). PGE_2 is a potent hyperalgesic agent and possesses synergistic effects with histamine and bradykinin which then excite the pain nerve ending in the peritoneal cavity (Salmon and Higgs, 1987). PNQ-4482 and aspirin significantly inhibited the writhing response in mice. Aspirin and NSAIDs decrease the number of writhes by inhibiting enzyme cyclooxgenase, the essential enzyme in the synthesis of PGs, in peripheral tissues. (Fields, 1987). PGs are synthesized at the site of injury and can act upon the peripheral afferent terminal to facilitate afferent transduction and augment the inflammatory state (Yaksh, 1996). The results obtained from many inflammatory models in this study suggest the inhibitory effect of PNQ-4482 on PGs synthesis. The analgesic property of PNQ-4482 can also probably be due to the blockade of the effect or the synthesis and/or release of endogenous substances that excite pain nerve ending similarly to aspirin and other NSAIDs.

Most traditional tests of nociception, such as the tail-flick and hot-plate tests, are based on a phasic stimulus of high intensity. The nociceptive experience is short-lasting, and it is thus not possible to assess modulatory mechanisms that may be triggered by the stimulus itself. There is reason to believe that tonic pain is modulated differently in the central nervous system than the pain elicited by these short-lasting stimuli (Tjolsen *et al.*, 1992).

The formalin test is different from most models of pain in that it is possible to assess the way an animal responds to moderate, continuous pain generated by injured tissue. Because of this connection to tissue injury, it is believed that the test provides a more valid model for clinical pain than the tests with phasic mechanical or thermal stimuli (Dubuisson and Dennis, 1977; Abbott *et al.*, 1981,1982; Alreja *et al.*, 1984)

The formalin test consists of two distinct phases, possibly reflecting different types of pain (Dubuisson and Dennis, 1977; Hunskaar *et al.*, 1985; Hunskaar and Hole,

1987; Rosland, 1991; Tjolsen *et al.*, 1992). The first phase starts immediately after injection of formalin. It is probably due to direct chemical stimulation of nociceptors, (Dubuisson and Dennis, 1977; Hunskaar *et al.*, 1985; Tjolsen *et al.*, 1992) and experimental data indicate that formalin predominantly evokes activity in C fibers, and not in Aδ afferents (Heapy *et al.*, 1987; Tjolsen *et al.*, 1992). The second phase starts approximately 15-20 min after formalin injection and lasts for 20-40 min. The second phase seems to be due to peripheral inflammatory response partly mediated by PGs (Heapy *et al.*, 1987; Hunskaar and Hole, 1987). Experimental results have indicated that substance P and bradykinin participate in the early phase, while histamine, serotonin, PGs and bradykinin are involved in the late phase (Shibata *et al.*, 1989b).

The early phase can be inhibited by centrally acting analgesics (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 (indomethacin and naproxen), corticosteroids (dexamethasone and hydrocortisone), as well as the centrally acting analgesics (Hunskaar and Hole, 1987; Chen *et al.*, 1995; Elisabetsky *et al.*, 1995; Santos *et al.*, 1995). In the present study, it was shown that PNQ-4482 and aspirin produced antinociceptive effects in both phases of the formalin test, but markedly in the late phase. Data obtained from early phase tests suggest that the analgesic activity of PNQ-4482 is mediated via an inhibition on excitation of local nociceptors and/or an inhibition of mediators responsible for pain induction. The result in the late phase indicates the inhibitory effect of PNQ-4482 on the synthesis and/or release of inflammatory mediators, especially PGs.

The antipyretic activity of PNQ-4482 was investigated in yeast-induced hyperthermia in rats. Fever is the regulation of body temperature and requires a delicate balance between the production and loss of heat; the hypothalamus regulates the set point at which body temperature is maintained. In fever, this set point is elevated. Fever may be provoked by many stimuli, such as bacteria and their endotoxins, viruses, yeasts, spirochets, protozoa, immune reactions, several hormones,

medications and synthetic polynucleotides. These are commonly called exogenic pyrogens. Cells stimulated by exogenic pyrogens form and produce cytokines called endogenic pyrogens. Endogenic pyrogens centrally affect the thermosensitive neurons in the preoptic area of the hypothalamus and increase the production of heat and decrease in heat loss. The body temperature increases until it reaches the set point. (Bowman and Rand, 1980; Flier and Underhill, 1994; Sturtinova *et al.*, 1995; Roberts II and Morrow, 2001).

The most important endogenic pyrogens are IL-1 and cachectin, also called the tumor necrosis factor-α (TNF-α). They are produced especially by monocytes and macrophages but also by endothelial cells and astrocytes. Also the interferons (IFN) α, β and γ display pyrogenic activity (Flier and Underhill, 1994; Stvrtinova *et al.*, 1995).

After administration of an endotoxin in an experiment, the level of plasmatic TNF-α increases and fever occurs. Increased concentrations of IL-1 and TNF-α are also found in sepsis. The production of these cytokines is regulated by the positive feedback mechanism. Besides this, macrophages activated by IFN-γ may increase the production of IL-1 and TNF-α initially induced by other stimuli. In the hypothalamus, IL-1 and TNF-α trigger the synthesis of PGE_2 from the AA of cytoplasmic membranes of target cells. The precise mechanism by which PGE_2 resets the central thermostat is not known. Aspirin and the NSAIDs display antipyretic activity by inhibiting the cyclooxygenase, an enzyme responsible for the synthesis of PGE_2 (these antipyretics do not inhibit the production of TNF-α, or IL-1). Glucocorticoids work antipyretically by inhibiting the production of IL-1 and TNF-α, and by inhibiting the metabolic processes of AA (Flier and Underhill, 1994; Sturtinova *et al.*, 1995; Ushikubi, 2000, Amabeoku *et al.*, 2001).

The result from the present study showed that PNQ-4482 effectively reduced the rectal temperature of hyperthermic rats, which indicates its antipyretic effect. The mechansim in reduction of fever by PNQ-4482 seems to be similar to that of aspirin and NSAIDs, since it was proved in previous inflammatory models that PNQ-4482 possessed an inhibitory effect on the biosynthesis of PGs. Certainly, its antipyretic effect should not

be similar to glucocorticoids, since PNQ-4482 did not possess steroid-like activity as shown in the model of cotton pellet-induced granuloma formation in rats.

The common major problem of using classical NSAIDs in clinical practice is their side effects on the gastro-intestinal tract. Gastric and duodenal ulcers are prevalent in the long term use of NSAIDs (Vogin and Rossi, 1961; Parmar and Ghosh, 1978; Gambhir *et al.*, 1987).

Peptic ulcers are caused when the natural balance between the aggressive factors of acid and pepsin and defensive mechanisms of mucus, bicarbonate, mucosal turnover and blood supply (mucosal barrier) is disturbed (Piper and Stiel, 1986). Acid and pepsin are relatively less important as causative agents and a defect in the defensive mechanism of gastric mucosa is the first step towards ulcer formation. The mucosal barrier is normally impermeable to back diffusion of hydrogen ions (McGuigan, 1980), but it is weakened by either decreased mucus secretion or by a disturbance in the turnover of epithelial cells caused by stress, corticotropin, cortisone, aspirin, phenylbutazone, ingestion of chillies, tobacco smoking, etc. (Croft, 1977; Menguy and Masters, 1965). Because of the varied ulcer pathogenesis, various types of antiulcer drugs have come into use to either overcome the acid and pepsin secretion (e.g. antacids and histamine H_2-blocking drugs) or to enhance mucus secretion and stabilize the surface epithelial cells (e.g. sucralfate, which binds to the proteinaceous material in the ulcer crater and prevents further digestion of the mucosa by acid and pepsin) (Spiro, 1982). These drugs have decreased morbidity and mortality but may also produce many adverse reactions, such as arrhythmias, impotence, gynaecomastia, haematopoeitic changes and high recurrence rates (Ariyoshi *et al.*, 1986).

Many attempts have been made to develop drugs which would retain anti-inflammatory activity and yet be devoid of these ubiquitous adverse effects. Some success has been achieved in this direction e.g. gossypin, a bioflavonoid isolated from *Hibicus vitifolius* Linn. (Parmar and Ghosh, 1978), curcumin from *Curcuma longa* Linn. (Merhra *et al.*, 1984; Ageel *et al.*, 1987), a hydroalcoholic extract and partitioned fraction

from *Turnera ulmifolia* (Antonio and Souza Brito, 1998), Cauvery-100 (an ayurvedic formulation), eugenol and ginger oil from *Zingiber officinale* Roscoe. (Manonmani *et al.*, 1994; Sharma *et al.*, 1994) as well as kolaviron, a biflavonoid extract from *Garcinia kola*, (Bradide, 1993; Ibriroke *et al.*, 1997).

Preliminary screening of PNQ-4482 showed that it did not possess any ulcerogenic activity when orally given for 7 days in an equal dose of aspirin. It was therefore of interest to further investigate the anti-ulcerogenic effect of PNQ-4482 by testing in some ulcerogenic models in rats such as ethanol/hydrochloric acid, indomethacin, restraint water immersion stress-induced gastric lesions and pylorus ligation models.

The ethanol/hydrochloric acid-induced gastric lesions model (Mizui and Doteuchi, 1988) used in this present study is an analytical method in which drugs that activate gastric mucosal protective factors are often effective (Yamahara *et al.*, 1990). The ethanol-induced gastric lesions are also known to be acid-independent (Robert, 1979). Thus, ethanol was orally given to rats together with sufficient acid to eliminate the influence of endogenous acid on gastric lesion (Yoshida *et al.*, 1999). Many factors have been reported to play an important role in gastroprotective functions such as mucosal blood flow, gastric mucus secretion and alkaline secretion. Among them, gastric mucosal blood flow appears to play a major role in gastroprotection through supplying nutrients and oxygen (Chenung and Ashley, 1987) and to counteract the disruption of gastric mucosal barrier and acid back-diffusion (Ritchie, 1975; Stalinger *et al.*, 1981). Recently, it was indicated that endothelium-derived nitric oxide (NO) as well as endogenous prostanoids may play an important role in mucosal microcirculation and mucus synthesis (Tepperman and Whittle, 1992). The aggravation of acidified ethanol-induced gastric lesion may be due to the decrease in gastric mucosal blood flow mediated by the decrese in the release of NO from endothelial cells (Ito *et al.*, 1996). The decrease in the gastric mucosal blood flow may cause aggravation of the gastric lesion by a decrease in gastric mucosal resistance and the disturbance of washing

away via the blood flow of a large amount of H^+ diffused into the mucosa from the gastric lumen after the administration of acidified ethanol (Yoshida *et al.*, 1999).

The ability of PNQ-4482 to protect gastric wall mucus against ethanol may be due to an increased mucosal resistance or potentiation of defensive factors against ethanol damage. The PGE_2 did not completely prevent the ethanol-induced damage to surface epithelial cells. Therefore, the product of the 5-lipoxygenase pathway may also play a key role in the development of this ulcer (Lacy and Ito, 1982; Schmidt *et al.*, 1985; Tarnawski *et al.*, 1985). It has also been reported that leukotriene antagonist and 5-lipoxygenase inhibitors are capable of inhibiting ethanol and NSAIDs-induced gastric ulceration in rats (Parnaham and Brune, 1987). Hence, the protective action of PNQ-4482 against ethanol/hydrochloric acid-induced gastric lesions could possibly be due to inhibition of 5-lipoxygenase pathway. This inhibitory effect on the leukotriene synthesis of PNQ-4482 was proved in the AA-induced ear edema model, which is the model widely used for the discovery of lipoxygenase inhibitors.

NSAIDs like aspirin and indomethacin are known to induce gastric ulceration. NSAIDs such as indomethacin are known to induce ulcer during the course of their anti-inflammatory action. NSAIDs inhibit COX enzyme leading to reduction of PG synthesis from arachidonic acid. Two isoforms of COX are COX-1, which is a constitutive enzyme, and COX-2, which is rapidly inducible and is responsible for the production of pro-inflammatory PGs (Andrews and Goldman, 1998). High concentrations of PGs, especially PGE_2 and PGI_2, are present in the normal gastric and duodenal mucosa and they are responsible for mucous production. Inhibition of PG synthesis by classical NSAIDs, such as indomethacin, which non-selectively inhibit both COX-1 and COX-2, causes gastric and intestinal ulceration and delays gastric ulcer healing in chronic ulcer. Selective COX-2 inhibitors represent a new pharmacological class of NSAIDs with minimal gastrointestinal toxicity. PGs, especially PGE_2 and their analogs, inhibit the formation of gastric mucosal necrosis induced by such necrotizing agents including NSAIDs (Robert *et al.*, 1983).

This principle pathway of inhibition of biosynthesis of "cytoprotective prostaglandin" e.g. PGE_2 and PGI_2 results in over production of leukotrienes and other products of 5-lipoxygenase pathway (Rainsford, 1987). It is well known that indomethacin decreases mucosal protective activities such as blood flow, mucus secretion and bicarbonate secretion and increases aggressive factors such as motility, increase gastric secretion. Indomethacin first stimulates the neutrophils and eosinophils to release bioactive substances such as major basic proteins, LTs, PAF and cytokines, which are related to inflammation (Gleich and Adolphson, 1986; Moqbel *et al.*, 1994). Parnaham and Brune (1987) reported that leukotriene antagonists and 5-lipoxygenase inhibitors are capable of inhibiting ethanol and NSAIDs-induced gastric ulceration in rats. In addition, It has been reported that the erosions induced by indomethacin were prevented by acid antisecretory agents such as H_2-receptor antagonists (Kuratani *et al.*, 1992; Yamahara, 1992).

PNQ-4482 significantly inhibited gastric ulcer induced by indomethacin. The results suggest that the anti-ulcerogenic activity of PNQ-4482 is probably associated with increases of defensive factors such as gastric mucus and microvascular blood flow and could possibly be due to inhibition of the 5-lipoxygenase pathway.

Stress factors, which may be in part psychological or physical, may exert an influence on the central nervous system, resulting in alternations in the limbic system which controls emotions and has a close connection with the hypothalamus as a regulatory center of the autonomic nervous system (sympathetic and parasympathetic) and the neuroendocrine system. In addition, pathophysiological changes in these areas are frequently involved in gastric acid hypersecretion (Brodie and Hanson, 1960; Kitagawa *et al.*, 1979), disturbances in gastrointestinal motility (Goldman and Rosoff, 1968), and increase of endogenous substances such as glucocorticoids, catecholamines and histamine. These alterations are considered to cause systemic arterial hypertension and disturbed microcirculation in the gastric mucosa, and, consequently, may lead to local ischemia and anoxia. More recently, considerable attention has been paid to the pathogenetic role of oxygen-derived free radicals (Itoh

and Guth, 1985; Perry *et al.*, 1986). All these functional and morphological changes may decrease the gastric mucosal resistance and vitality of the capillary cells against the aggressive factors (psychological, physical and chemical), eventually leading to necrosis, erosion, hemorrhages and ulcerations (Yabana and Yachi, 1988).

Stress-induced ulcers are probably mediated by histamine release with enhancement in acid secretion. (Goa and Monk, 1987). Eagleton and Watt (1971) have reported that the vascular disturbances caused by histamine may be the main etiological factor of gastric lesions in the case of histamine-induced ulcer. In this study PNQ-4482 exhibited an inhibitory effect against cold stress-induced ulcer. Therefore, PNQ-4482 may inhibit histamine release, thereby causing a reduction in ulcer formation. This postulation of inhibitory effect of PNQ-4482 on histamine release and/or synthesis has also been proved in carrageenin-induced rat hind paw edema.

The pylorus ligation model is used for evaluation of anti-secretory activity (Shay *et al.*, 1945). This model has been shown to stimulate acid secretion via the vago-vagal reflex and cholinergic muscarinic mechanism which cause accumulation of intraluminal HCl (Shay *et al.*, 1945; Brodie, 1966; Hakanson *et al.*, 1980). Cimetidine reduces both the volume of gastric juice secreted and its H^+ concentration (Brunton, 1996). In this present study, cimetidine caused significant decreasing of both gastric volume and total acid output. Antonio and Souza Brito (1998) suggested that agents which exhibit an anti-ulcerogenic activity in this model could act through histamine inhibition. Administration of PNQ-4482 produced a significant decrease of gastric volume and total acid output. It is therefore suggested that PNQ-4482 may possess an inhibitory activity on histamine release and /or synthesis, thereby reducing secretory activity.

A Hippocratic screening test of PNQ-4482 has been performed in the present study. The Hippocratic screening test is commonly used in the preliminary screening of medicinal plants to detect interesting pharmacological activities (Malone and Robichaud, 1962). Since a single administration of PNQ-4482 by the oral route up to a dose of 5000 mg/kg did not exhibit any mortality or produce any sign of toxicity in rats,

the test substance was then given by an intraperitoneal route. Two doses levels, an ineffective and lethal dose, were first determined. The three effective doses were calculated according to the equation in the Hippocratic screen. The intensity of responses increased with the increasing doses. Signs and symptoms which occurred in response to a high intraperitoneal dose of PNQ-4482 were a decrease in motor activity, loss of screen grip, loss of righting reflex and decrease of respiratory rate. These effects therefore suggest a central nervous system depressant activity or sedative activity (Malone and Robichaud, 1962). Sign of respiratory failure was observed before death in rats which received the dose of 3,900 mg/kg. No observable sign of abnormalities of the internal organs could be detected and they were found to be normal in both size and color. The results of the Hippocratic screening test indicate that only a high dose of PNQ-4482, given intraperitoneally, possesses a depressant effect on the central nervous system.

The acute toxicity test is used to determine the degree of toxicity of a chemical substance, that is, the relationship between dose and adverse effects; to establish its toxicity relative to other chemical substances whose acute toxicity is known; to determine specific toxic effects; and to provide information on the mode of toxic action. By studying the effects, following administration by different routes, the relative hazards of different pathways of exposure can be assessed. By using animals of both sexes, sex differences in a toxic response can be detected. An acute toxicity study will thus identify highly toxic chemicals and provide information on the possible hazards which could occur where humans are exposed (OECD guidelines for testing of chemicals, 1981).

In this present study, an acute toxicity study of PNQ-4482 has been performed. A single administration of PNQ-4482 by the oral route up to a dose of 5000 mg/kg did not produce any mortality or show any signs of toxicity or changes in general behavior or other physiological activities when compared with the control group. According to the OECD guideline for testing of chemicals the results of acute toxicity test in this study indicate that PNQ-4482 is fairly non-toxic.

While acute toxicity deals with the adverse effects of single doses, a more common form of human exposure to many chemical substances is in the form of repeated doses which do not produce immediate toxic effects. Delayed effects may occur due to accumulation of the chemical in tissues or to other mechanisms and it is important to identify any potential for these by a subacute toxicity test. The main study durations involve 14, 28, and 90 days. Other study durations have been used in toxicological investigation but the selection of three primary durations, which have either the backing of experience or existing regulatory requirements, is considered to represent a reasonable approach. These studies will provide detailed information on toxic effect, target organs, reversibility or otherwise of effect and an indication of a "no effect level". It is recommended that the use of a satellite group of test animals, given the highest dose and then observed after the ending of dosing, be considered in order to give additional information on the persistence or reversibility of effects (OECD guidelines for testing of chemicals, 1981).

This present work indicates that administration PNQ-4482 at a dose of 1,000 mg/kg daily for 14 days did not produce any significant changes in body and organ weights, hematology, gross appearance and histopathology in treated rats. However, the weights of liver and spleen of the male satellite group were significantly less than those of the control group. This effect on organ weight (liver and spleen) may be because of the organ size and/or weight variation of animals in the male satellite group.

Serum albumin levels regularly decline severely in patients with severe hepatocellular disease. In addition, liver disease may accompany any of these other problems (malnutrition, protein-losing alimentary tract disease, protein-losing renal disease, prolonged severe catabolic conditions like burns and in condition of expanded blood volume), making it difficult to assess the relative significance of the single laboratory finding without associated tests whose values may form diagnostic patterns of abnormalities (Sacher and McPherson, 1991). From the blood chemistry of animals in this study, the albumin value of the female satellite group was significantly less than that of the control group but nevertheless not a severely depressed value and not related to

other liver parameter indexes i.e. total protein, total bilirubin, direct bilirubin, SGOT, SGPT and alkaline phosphatase. Decreased albumin levels are caused by many different conditions i.e. inadequate iron intake, severe liver diseases, malabsorption, diarrhea, eclampsia, nephrosis, exfoliative dermatitis, third-degree burns, starvation and excessive administration of intravenous glucose in water. It is therefore suggested that PNQ-4482 may not affect liver function of the female satellite group.

Alkaline phosphatase levels reach spectacular heights (up to 20 folds of normal values) in primary biliary cirrhosis, in conditions of disorganized hepatic architecture (cirrhosis), and in diseases characterized by inflammation, regeneration and obstruction of intrahepatic bile ductules. In contrast, significant decrease of alkaline phosphatase does not indicate any abnormality of liver function (Sacher and McPherson, 1991). Therefore a decrease of alkaline phosphatase in the male satellite group in this study, does not suggest any abnormality of liver function of the animals.

Blood creatinine becomes very highly elevated when renal function declines. If slow loss of renal function occurs simultaneously with slow loss of muscle mass, the concentration of creatinine in serum may remain stable, but 24-h excretion (or clearance) rates would be lower than normal. This pattern may happen in aging patients. Thus a better index of renal function is creatinine clearance, which takes into account both serum creatinine and the quantity excreted in a day (Sacher and McPherson, 1991). In this study, the male satellite group showed a significant rise of blood creatinine but still not higher than 1 fold when compared with that of the control group. Normally not only blood creatinine is used as an index to detect the renal function but also blood urea nitrogen, creatinine clearance, osmolality of urine, specific gravity of urine and urine concentration. Thus, the slight increase in blood creatinine of the male satellite group does not exactly suggest that PNQ-4482 affects the renal function.

In conclusion, the results of the present study show the anti-inflammatory, analgesic and antipyretic activity of PNQ-4482. The anti-inflammatory effect of PNQ-4482 was found prominently on the acute phase of inflammation. It is likely that PNQ-

4482 reduces inflammation by inhibiting both the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism and other mediators e.g. histamine, serotonin etc. In the chronic inflammation, PNQ-4482 slightly inhibits the transudative phase and fibroblast proliferation. Moreover, PNQ-4482 appeared to be devoid of steroidal like effects such as causing marked decrease of the normal body weight gain and the thymus weight. PNQ-4482 reduced the alkaline phosphatase activity in serum, which might be due to its stabilization activity on lysosomal membrane of leukocyte. The analgesic and antipyretic activity of PNQ-4482 might be mediated by inhibition of PG-biosynthesis and other mediators. PNQ-4482 did not produce gastric ulcer as compared with aspirin. In addition, PNQ-4482 showed anti-ulcerogenic activity in the experimental models of gastric lesions induced by ethanol/hydrochloric acid, indomethacin, restraint water immersion stress. Furthermore, PNQ-4482 reduced the gastric volume and total acid output. It can be postulated that the anti-ulcerogenic effect of PNQ-4482 is due to an increase of defensive factors such as gastric mucus and microvascular blood flow through inhibiting the release and/or synthesis of histamine and could possibly be due to an inhibition of 5-lipoxygenase pathway as well as possessing anti-secretory effect. In the Hippocratic screen, acute toxicity and subacute toxicity studies with PNQ-4482 did not show any signs of toxicity when assessment was made from both gross and histopathological examination.