DISCUSSION

Inflammation is a complex process and various mediators have been reported to be involved in the development of inflammatory diseases (Yesilada *et al.*, 1997). This fact, associated with the complexity of the inflammatory process, resulting in employment of different experimental models is essential when conducting pharmacological trials (Singh *et al.*, 1989). More understanding about the inflammatory response and its underlying mechanism is needed, and this will undoubtedly lead to advances in this field (Handa *et al.*, 1992). The search by screening and development of drugs for anti-inflammatory activity is an unending problem. There are lots of research work on indigenous plants with the hope of finding active anti-inflammatory compounds, as the people in the third world still use medicinal plants in therapeutics despite the progress made in conventional chemistry and pharmacology for producing synthetic drugs (Handa *et al.*, 1992).

The present study on *Diospyros variegata* Kruz. was carried out because it has been used in Thai folklore medicine for management of pain and inflammatory conditions and some species of *Diospyros* are widely used for different types of inflammatory diseases (Pongboonroud, 1976; Mallavadhani *et al.*, 1998). Furthermore, the previous study for anti-inflammatory activity by Trongsakul (1999) showed that the hexane extract from the stem of *D. variegata* possessed marked inhibitory effect on the edema formation on ethyl phenylpropiolate (EPP) and arachidonic acid-induced ear edema in rats. Therefore, it is of interest for further investigation of its anti-inflammatory activity in various animal models which can be mimic a broad spectrum of acute and chronic inflammation.

It is known that the acute inflammatory response consists of three main vascular events: (i) vasodilatation and increased vascular flow, (ii) increased vascular permeability, and (iii) leukocyte migration to the injured tissues (Antonio and Souza Brito, 1998). The carrageenin-induced inflammation of the rat paw is a

test which has been frequently used to assess the anti-edematous effect of natural products (Mascolo *et al.*, 1987). 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 development of edema in the paw of the rat after the injection of carrageenin was described by Vinegar *et al.* (1969) as a biphasic event. The initial phase seen at the first hour is attribute to the release of histamine, kinins and serotonin (5-HT). The edema maintained during this phase is presumed to be due to kinin-like substances, the time course of the activation of this system is not clearly known (Gryglewski, 1977). The second phase of swelling is due to the release of prostaglandins (PGs), occurred 1-3 h after carrageenin injection and lasted about 7 h (Di Rosa *et al.*, 1971). All of mediators are capable of promoting vasodilatation and increasing vascular permeability as well as synergistically producing edema (Carlson *et al.*, 1985). It has been reported that the second phase of edema is sensitive to both clinically useful steroidal and nonsteroidal anti-inflammatory agents (Vinegar *et al.*, 1969; Di Rosa *et al.*, 1971). The significant inhibitory effect of DVHE and aspirin (a non-selective cyclooxygenase inhibitor) on carrageenin-induced paw edema at the second phase suggests that the main mechanism of action of DVHE may involve the PG biosynthesis and/or release. DVHE 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 suggest that DVHE possesses an anti-inflammatory activity, likewise aspirin, by inhibition of the cyclooxygenase pathway and other inflammatory mediators of the acute phase of inflammation. The results in this test model support the possibility of mechanism of action of DVHE on the cyclooxygenase pathway and on other inflammatory mediators, which was suggested in ear edema caused by EPP (Trongsakul, 1999). According to the

percent inhibition on hind paw edema of DVHE at the dose of 300 mg/kg, it was found that the intensity of the anti-edematous effect was less than that of aspirin at the same dose. As DVHE is the crude extract which contains numerous different groups of substances, continuing phytochemical work on this extract may give the information of active constituent(s) possessing higher anti-edematous effect than the crude one.

Injection of irritants into enclosed body cavities provides a cell rich fluid exudate for sampling. Originally the peritoneal cavity was used but because of its size and the presence of the gastrointestinal tract, quantification of fluid volume and composition is difficult. The most suitable cavity for studying the inflammatory response was found to be the pleural cavity (Sedgwick and Willoughby, 1989). 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). 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). Furthermore, the fluid accumulation in response to intrapleural injection of carrageenin occurs in parallel with the release of prostaglandin E_2 (PGE_2) and the simultaneous production of bradykinin (Katori *et al.*, 1978). Lo *et al.* (1981) reported that PGs might act in potentiating the effect of permeability-increasing mediators (kinins, histamine and serotonin). Mikami and Miyasaka (1983) suggested that the reduction of PGE_2 contents might be the main contribution to the anti-exudative activities of anti-inflammatory drugs in rat carrageenin pleurisy. The present study demonstrates that the steroidal anti-inflammatory drug (prednisolone), nonsteroidal anti-inflammatory drug (aspirin) and DVHE elicited significant reduction of the pleural exudative formation at the 3^{rd} and 6^{th} hour after carrageenin injection. Flower and Dale (1989) reported that the steroidal anti-inflammatory drug such as

prednisolone induce the synthesis of a protein inhibitor of phospholipase A_2 namely lipocortins, as a result inhibition of prostaglandin biosynthesis. In addition, they also decrease capillary permeability by reducing the amount of histamine released by basophils and mast cells (Chrousos and Margioris, 2001). Therefore they have potent anti-inflammatory effects in this model. 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 results of present study suggest that DVHE probably possess anti-exudative effect, likewise aspirin and other NSAIDs, by reducing the PGs production. For a better understanding of the mechanism of action of DVHE, bioassay following the method described by Willis (1969) for measuring prostaglandin-like activity of the exudate using rat fundus strip should be performed.

Vannier *et al.* (1989) suggested the three factors involved in the *in vivo* leukocyte migration, i.e. extravasation of plasma proteins, generation of chemotactic factors related to complement components and leukocyte chemotactic responsiveness. Leukocyte accumulation into the pleural cavity in response to carrageenin depends partly on the activation of the complement system (Capasso *et al.*, 1975; Sedgwick *et al.*, 1985). 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 leukocytes elicited by carrageenin were constituted by neutrophils (Vannier *et al.*,1989). Tarayre and Lauressergues (1980) found that the leukocytes in the pleural exudate are neutrophils, eosinophils, macrophages and lymphocytes. The percent of these cells are 81, 0.5, 16 and 2.5 respectively. 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. 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; and Furst and Munster (2001) found that aspirin inhibits the migration of polymorphonuclear leukocytes and macrophages into the site of inflammation. On the contrary, the result of the present study showed that DVHE exhibited a nonsignificantly inhibitory effect on leukocyte accumulation. This result suggests that DVHE rather possesses anti-exudative effect than inhibitory effect of leukocyte accumulation. From this result it may be postulated that the dose of DVHE used for this study (on the inhibition of leukocyte accumulation) may be too low. Further study of higher dose of DVHE is warranted to exhibit significant inhibition of leukocyte accumulation. Vannier *et al.* (1989) reported that the volume of exudate is more inhibited by NSAIDs such as indomethacin and ketoprofen than the number of leukocytes. At doses that suppress the prostaglandin synthesis *in vivo*, indomethacin has very few effects on the leukocyte migration (Walker *et al.*, 1976).

Parente *et al.* (1979) and Lo *et al.* (1984) reported that indomethacin either inhibit the *in vivo* leukocyte migration by reducing extravasation of plasma proteins without impairing the subsequent generation of chemotactic factors or reduce the chemotactic responsiveness of leukocytes to these chemotactic factors. From this research, the results suggest that DVHE exhibit inhibition of leukocyte accumulation in the same way likewise indomethacin and other NSAIDs.

Iwamura *et al.* (1984) reported that the steroidal anti-inflammatory drugs show an anti-exudative effect throughout the period of testing after carrageenin which more marked in the relatively late phase than in the relatively early phase. On the contrary, the nonsteroidal anti-inflammatory drugs show anti-exudative effect only in the early phase and not thereafter. The present result showed that the inhibitory activity of prednisolone on the exudate formation at the 6^{th} hour was

greater than that found at the 3rd hour. In addition, the inhibitory activity of aspirin and DVHE on the exudate formation at the 6th hour was less than that found at the 3rd hour. The result obtained suggests the difference in mechanism of anti-inflammatory action between DVHE and prednisolone.

The cotton pellet-induced granuloma formation is a typical feature of established chronic inflammatory reaction and can be served as a subchronic and chronic inflammatory test model for investigation of anti-arthritic substances (Spector and Willoughby, 1959). It has been widely employed to assess the transudative and proliferative component of chronic inflammation. The fluid adsorbed by the pellet greatly influences the wet weight of the granuloma and the dry weight correlated well with the amount of granulomatous tissue formed (Swingle and Shideman, 1972). The effects of DVHE, aspirin and prednisolone on the transudative and the proliferative phase of chronic inflammation were determined. Prednisolone, a steroidal anti-inflammatory drug, has catabolic effect in connective tissue, inhibits the function of leukocytes and tissue macrophages and decreases capillary permeability (Chrousos and Margioris, 2001). In addition, they decrease proliferative response to mitogens and antigens which has been shown to be associated with decrease production of interleukins-2 (T cell growth factor)(Flower and Dale, 1989). Therefore it exerted marked inhibitory activity on the formation of transudate and granuloma. The effects of DVHE and aspirin on the proliferative phase seem to provide evidence for the suggestion of Winder *et al.* (1962; 1965) that the effect of most nonsteroidal anti-inflammatory agents on granuloma formation is slight. It is suggested that DVHE does not possess an anti-inflammatory activity on chronic inflammation likewise aspirin and other nonsteroidal anti-inflammatory agents.

Arachidonic acid metabolites, particularly leukotriene B_4 (LTB_4), could 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). Neutrophils and monocytes contain lysosomal granules, which when released may contribute to the inflammatory response (Collins, 1999). Alkaline phosphatase is contained within leukocytes in specific granules (Arrigoni-Martelli and Restelli, 1972). The role of lysosomal enzymes such as alkaline phosphatase as mediators of inflammation is well documented (Becker and Henson, 1973). The activity of alkaline phosphatase raised in serum during inflammatory process, results in the damage of tissue and cartilage that can lead to further perpetuation of the inflammation (Bessey *et al.*, 1946). The leakage of lysosomal content into the extracellular space has been widely considered as a critical pathway along which various inflammatory processes proceed (Weissmann, 1965b, 1966, 1967; Weissmann and Spilberg, 1968). It is known that 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). Agents that can prevent the rupture of the lysosomal membrane and thereby prevent damage to the tissue caused by the release of the hydrolytic enzymes contained within the lysosome may be expected to alleviate some symptoms of inflammation (Hess and Milonig, 1972). Weissmann (1965a) reported that few agents that stabilize the membranes of lysosomes have been found. Chief among these are the anti-inflammatory steroids: cortisone, cortisol, prednisone, prednisolone, beta-methasone (as acetates, phosphates or hemisuccinates) but not corticosterone. Das and Dasgupta (1997) and Ebong *et al.* (1998) reported that aspirin significantly decrease the activity of alkaline phosphatase. 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 DVHE. This result suggests the efficacy of DVHE and reference drugs in stabilizing the lysosomal membrane during inflammation. Beside this, DVHE was proved in arachidonic acid-induced ear edema model to inhibit lipoxygenase

pathway in producing LTs (Trongsakul, 1999). This effect, especially by inhibiting LTB_4 biosynthesis, can reduce leukocyte migration into inflammatory area and thereby reducing lysosomal enzyme leakage from those cells. From this result it may be postulated that the continuous administration of DVHE may correlated with inhibition of leukocyte migration in chronic inflammation.

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 glucocorticoids such as prednisolone, stimulate protein synthesis in liver, they have pronounced catabolic effects in lymphoid and connective tissue, muscle, fat and skin. In fat metabolism they increase lipolysis through a "permissive" effect on the action of other lipolytic hormones. Supraphysiologic amounts of glucocorticoids lead to decreased muscle mass and weakness (Flower and Dale, 1989; Chrousos and Margioris, 2001). The results obtained confirm the difference in mechanism of anti-inflammatory action of DVHE and prednisolone since DVHE did not influence the body and the thymus weight.

It is generally accepted that peptic ulcers are caused by a disruption in the balance of aggressive factors and mucosal defensive factors. Aggressive factors are acid and pepsin, *Helicobacter pylori* infection and NSAIDs. Defensive factors are gastric mucus and bicarbonate, gastric mucosal barrier and blood flow and PGs (Morimoto *et al.*, 1991; Friedman and Peterson, 1998). NSAIDs, e.g. acetylsalicylic acid, phenylbutazone and indomethacin, are known to induce ulcers during the course of their anti-inflammatory action (Pal and Nag Chaudhuri, 1991). These drugs induce gastric lesions by inhibiting cyclooxygenase (COX) (Brooks and Day, 1991). The two isozymes also differ in function in that COX-1 is widely distributed and has "housekeeping" functions, e.g. gastric cytoprotection. In contrast, COX-2 is an immediate early response gene product in inflammatory and immune cells (Foegh and Ramwell, 2001). The toxicity of NSAIDs in the gastrointestinal (GI) system is thought to be related to the lack of selectivity of

those drugs with respect to inhibition of COX-1 and COX-2 (Vane, 1994). Research on these drugs to eliminate this side effect has been intensified in recent years (Smith *et al.*, 1994; Vane and Botting, 1995). Ideal anti-inflammatory drugs should have an inhibitory action on PGs synthesis mediated by COX-2, but not by COX-1 (Smith *et al.*, 1994). From this reason, the effect of DVHE on the stomach was compared to that of aspirin. In this study, it is found that DVHE did not induce gastric lesions when compared to aspirin. The study of the effect of DVHE on COX-1 and COX-2 from the laboratory in Uppsala University, Sweden, showed that DVHE inhibited both COX-1 and COX-2 with the percentage of inhibition of 73.04 and 50.2, respectively. The COX-2 : COX-1 inhibition ratio of DVHE was found to be 0.7 (Trongsakul, 1999). Meloxicam, a selective COX-2 inhibitor, with lower side effects on gastrointestinal tract and on kidney function, showed selectivity in the whole cell assay for COX-2 and 75-fold selectivity for COX-2 in the microsomal assay. In intact animal experiments, meloxicam showed a very favourable COX-2 : COX-1 ratio of 0.8 (Frolich, 1997). The result suggests that DVHE may act as a selective COX-2 inhibitor likewise meloxicam. It is therefore of interest for further investigation of anti-ulcerogenic effect, as the plant of the same genus, i.e. *D. paniculata* is used to treat rheumatism and ulcers (Mallavadhani *et al.*, 1998). Therefore, DVHE was further tested in the indomethacin-induced gastric lesion and pylorus ligation models.

Indomethacin is a prostaglandin inhibitor which suppress gastroduodenal bicarbonate secretion, disrupts the mucosal barrier, reduces endogenous prostaglandin biosynthesis as well as gastric mucosal blood flow (Whittle, 1977; Flemstrom *et al.*, 1982; Miller, 1983; Selling *et al.*, 1987). On the other hand, prostaglandins synthesized in large quantities by the gastrointestinal mucosa are known to prevent experimentally induced ulcers caused by various ulcerogens; and Robert *et al.* (1983) has discussed the role of prostaglandins in cytoprotection. Mucus secretion in the stomach and small intestine is increased by PGEs. These effects help to maintain the integrity of the gastric mucosa and are referred

to as the cytoprotectant properties of PGEs (Campbell and Halushka, 1996). Robert *et al.* (1979) coined the term "cytoprotective" to describe the property of PGs (and other compounds which have no structural similarity with PGs) by which cells are rendered defensive to stave off gastric mucosal lesions induced by various necrotizing agents such as ethanol, strong acid or base, and NSAIDs. This pharmacological action is achieved independently of the inhibition of acid and secretion. Furthermore, PGEs and their analogs inhibit gastric damage caused by a variety of ulcerogenic agents and promote healing of duodenal and gastric ulcers (Campbell and Halushka, 1996). Misoprostol (Cytotec), a methyl analog of PGE_1, is currently approved for the prevention of gastric ulcer disease induced by NSAIDs in patients who must take aspirin-like drugs and who are at high risk for complicated gastric ulcer disease (Brunton, 1996). The result of the present study showed that DVHE possessed only a nonsignificant anti-ulcerogenic activity in this model. It is suggested that DVHE lacks cytoprotective activity.

The pylorus ligation model is used for evaluation of anti-secretory activity (Shay *et al.*, 1945). In this present study, cimetidine exerted anti-secretory activity causing significant decrease of both the gastric volume and the total acidity. On the contrary, DVHE is found to be devoid of an anti-secretory activity since it did not affect the gastric volume and the total acidity. Cimetidine, a H_2 receptor antagonist drug, inhibits the gastric acid secretion elicited by histamine and other H_2 agonists. It also inhibits gastric acid secretion elicited by gastrin and, to a lesser extent, by muscarinic agonists. Cimetidine reduce both the volume of gastric juice secreted and its H^+ concentration (Brunton, 1996). Antonio and Souza Brito (1998) suggested that agents, which exhibit an anti-ulcer activity in this model could be due to histamine inhibition. It is therefore suggested that DVHE does not possess an anti-histaminic activity and therefore lack of anti-secretory activity.

In conclusion, the results of the present study show the anti-inflammatory of DVHE in acute inflammation and some activity in chronic inflammation. The anti-inflammatory effect of DVHE was found prominently on acute inflammation.

In acute inflammation, DVHE exhibit anti-edematous and anti-exudative activity. It is likely that DVHE reduces inflammation by blocking both the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism. The inflammatory activity of DVHE might be mediated by blocking PG biosynthesis, especially PGE_2, and the other mediators e.g. histamine and 5-HT. In chronic inflammation, DVHE did not inhibit the transudative phase and fibroblast proliferation. Moreover, DVHE appeared to be devoid steroidal like effects such as causing marked decrease of the normal body weight gain and the thymus weight. However, DVHE reduced the alkaline phosphatase activity in serum. The inhibition of lysosomal enzyme (alkaline phosphatase activity) might be due to lysosomal membrane stabilization which can prevent tissue and cartilage damage in chronic inflammatory diseases. In contrast, the administration of DVHE did not potentiate gastric mucosal lesions when compared with aspirin. The anti-inflammatory action of DVHE could be postulated to be due to an inhibitory effect on COX-2 rather than COX-1. Moreover, DVHE did not inhibit the appearance of gastric mucosal lesions induced by indomethacin and did not exhibit anti-secretory activity in pylorus ligature. Further studies both in phytochemistry and pharmacology should be conducted to find out the active principles to be potential candidates as novel safe therapeutic agents for the treatment of inflammatory diseases that is free from gastric intolerance.