DISCUSSION

Animal studies

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 correlate with clinical experience [57]. The edema induced in the rat hind paw by the local injection of carrageenin is mediated (like many other edematous processes) by the initial release of histamine and serotonin and is followed by the release of bradykinin during the 1st h after carrageenin injection, causing increased vascular permeability [68-69]. The second phase of inflammation is due to the release of prostaglandins. Prostaglandins play a major role in the development of the second phase of reaction which is measured around 3 h and lasts about 7 h after carrageenin injection [68-69]. The carrageenin-induced hind paw edema model in rats is known to be sensitive to COX inhibitors and has been used to evaluate the effect of NSAIDs which primarily inhibit the COX involved in prostaglandins synthesis. It has been demonstrated that the suppression of carrageenin-induced rat hind paw edema after the 3rd h correlates reasonably well with the therapeutic doses of most clinically effective anti-inflammatory agents [70].

The insignificant inhibitory effect of DJW on carrageenin-induced paw edema at the 3rd h, suggests that the main mechanism of action of DJW may not involved prostaglandins synthesis and/or release. Since DJW showed no inhibitory effect at the 1st h, it may not possess any influence on the other mediators, e.g., histamine and serotonin, which are released during this hour as well. In addition, the finding that oral DJW 2 and 4 h prior to carrageenin injection did not yield significant effects suggests that the ineffectiveness of DJW in this model does not correlate with the delayed absorption of DJW.

The inflammatory granuloma is a typical feature of established chronic inflammatory reaction and can serve for investigation of anti-arthritic substances [71-72]. The cotton pellet granuloma method has been widely used to assess the transudative and proliferative components

of chronic inflammation. The fluid, that is low in protein and is noninflammatory in origin, absorbed by the pellet greatly influences the wet weight of the granuloma and the dry weight correlates 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 occurs during the first 3 h, 2) an exudative phase, defined as leaking of plasma from the blood stream 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 3 and 6 days after implantation. Although the anti-inflammatory drugs can inhibit both the transudative and proliferative phases, NSAIDs give only slight inhibition, whereas, steroidal anti-inflammatory agents have a strong inhibition on both phases [58]. In the present study, DJW showed no effect on both transudative and proliferative effect. On the other hand, diclofenac, an NSAID, showed slight inhibitory activity on the formation of transudate and granuloma, whereas, prednisolone, a steroidal drug, exerted marked effect. Concerning 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 tissues, muscle, fat and skin. These results indicate that DJW has no anti-inflammatory and steroid-like activity in this chronic inflammatory model.

Arachidonic acid metabolites can mediate or modulate leukocyte influx into inflammatory sites, leading to tissue damage by releasing lysosomal enzymes and toxic oxygen radicals [73]. The activity of lysosomal enzymes, such as 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 serum alkaline phosphatase is elevated during cotton pellet granuloma formation, peaks on the 7^{th} day and decreases by day 14 when healing occurs [74-75]. Elevated lysosomal enzyme activity in serum and exudate during inflammation can be normalized by steroidal drugs, such as hydrocortisone, via the stabilization of lysosomal membrane [76]. In the present study, elevated serum alkaline phosphatase activity in this method was normalized by prednisolone only. This means that DJW and diclofenac may have no effect in stabilizing the lysosomal membrane during chronic inflammation.

Although some plants in DJW exhibit anti-inflammatory effects [15, 18, 28, 29, 32, 35, 46, 54], this study could not reveal these effects in both carrageenin-induced rat paw edema and cotton pellet-induced granuloma models. This might be because relative low dose (only 2.5-8%) of each ingredient was used. In addition, since DJW consists of many plants, the antagonism between the ingredients may lead to insignificant anti-inflammatory effect in this study.

The formalin test is different from most models of pain in that it is possible to assess the way 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 and reliable model for clinical pain than other tests of nociception [77-80]. It is a very useful method for not only assessing the analgesics but also elucidating the mechanism of pain and analgesia whether the site of action is central and/or peripheral [81]. The formalin test consists of two distinct phases, possibly reflecting different types of pain [59,77,82-83]. The early phase starts immediately after injection of formalin and lasts for 3-5 min. It is probably due to direct chemical stimulation of nociceptors [77,82-83], and experimental data indicate that formalin predominantly evokes activity in C fibers, and not in Aδ afferents [84]. This phase can be inhibited by centrally acting analgesics [59,82]. The late phase starts approximately 15-20 min after formalin injection and lasts for 20-40 min. It seems to be due to the combination of an inflammatory response in the peripheral tissue partly mediated by prostaglandins and functional changes in the dorsal horn of the spinal cord that were initiated by C fiber barrage during the early phase [83]. This phase can be inhibited by NSAIDs and steroids, as well as the centrally acting drugs [15,59]. Experimental results have indicated that histamine, serotonin and bradykinin are also involved in the late phase [81].

In the clinical study, DJW was prescribed at 3 g/dose, 3 times a day and we assumed that the mean body weight of the patients was 50 kg. So the dose used would be 60 mg/kg. If we subtracted the interspecies and intraspecies variation between human and rodent, the dose in rodent would be 6,000 mg/kg. From the preparation of the ethanol extract of DJW, % yield was 13.55. Thus, the dose of DJW extract that related to the dose used in clinical study became approximately 1,000 mg/kg.

In the formalin test, we used 1,000 mg/kg of DJW extract to verify the effectiveness of the dose that related to that used in clinical study. The higher (4,000 mg/kg) and lower dose (250

mg/kg) of DJW extract were also investigated to verify whether there were the relationship between doses and analgesic effect or not. These data might be used to apply for dose-adjustment in medical practice.

In the present study, codeine, diclofenac and all doses of DJW produced antinociceptive effects in both phases of the formalin test, but all drugs exerted marked effect in the late phase. Concerning the early phase, the reference drug, codeine exhibits its central analgesic effect by affecting the pain transmission and modulation pathway [84], whereas, diclofenac, affects via various possible mechanisms. The central action of diclofenac may be the result of a depression of C fiber-evoked activity or an inhibitory effect on central prostaglandin synthesis [85]. Alternatively, it may be mediated in part by endogenous opioid peptides or activation of descending serotonin pathways or even by a mechanism mediated by inhibition of excitatory amino acids or *N*-methyl-D-aspartate (NMDA) receptors [85]. For DJW, the mechanism(s) of central analgesic effect might be mediated via at least one possible mechanism similar to either codeine or diclofenac. A further study using an opioid antagonist, such as naloxone, in the formalin test should be done to investigate whether DJW exerts its effects via similar mechanism to codeine or not. In the late phase, the marked effect of all drugs may reflect an inhibition in both ongoing activity of nociceptors and central sensitization. In addition to central effect, diclofenac can inhibit COX leading to an inhibition of synthesis and release of prostaglandins that are considered to be the important inflammatory mediators involved in this phase. Concerning DJW that exerted its effect in a dose-dependent manner in this phase but showed a flat dose response curve in the early phase, this indicates that DJW might have some additional analgesic effect via anti-inflammatory mechanism. However, since DJW exhibited insignificant anti-inflammatory effect in carrageenin-induced rat paw edema, thus, the analgesic activity of DJW may be mediated via other pathways than COX pathway. The further animal studies using other anti-inflammatory models should be done to investigate these possibilities.

Clinical trial

In this study, the sample size calculation was determined by using the percentage of the responders in each group as the main efficacy criterion. The responder rate was initially estimated to be 80% and the efficacy of both treatments were assumed comparable if the

difference in response rate between the 2 groups was not more than 15% (delta < 0.15). The type I error (alpha) and type II error (beta) were 0.10 (one-tailed) and 0.20, respectively. On this basis, a number of 84 patients per treatment group were statistically necessary. With a projected dropout rate of 20%, 100 patients per treatment group were needed. From the results of this study, the number of completers in each group was greater than 84 patients and the responder rate in both groups were in line with our assumption. Therefore, the power of test in this study is believed to be adequate.

Since the preparations and dosages of DJW and diclofenac were different, this study was designed to be a randomized, double dummy, controlled trial in order to completely blind both patients and physician (double-blind). Therefore, the placebo of DJW was also prescribed to the patients in diclofenac group, and vice versa, the placebo of diclofenac was also prescribed to the patients in DJW group.

Among the 15 herbs used as raw materials in DJW, Xixin (Herba Asari) seemed to be the most toxic herb due to its pungent taste and warm property [21]. Generally, large dose of this herb is not recommended in the tropical country (such as Thailand) due to potential aggravation of internal heat. Thus, the amount of Xixin in DJW recipe used in this study was reduced from 7.75% to 2.5%.

The recommended dosage of DJW is 9-18 g/d [10-11], but in this study, the lowest dose (9 g/d) was chosen because we expected that the compliance with medication would be better and more acceptable than the higher doses. In addition, the lowest dose should be appropriate for the elderly whose efficiency of drug elimination (renal and/or hepatic function) might begin to be compromised. In the same manner, the low dose of diclofenac (75 mg/d) was also used in order to avoid or minimize its adverse effects, especially NSAIDs-induced gastropathy.

The DJW (500 mg/capsule) was prescribed as 6 capsules 3 times a day instead of larger doses and less frequent dosing interval (e.g., 9 capsules 2 times a day or 18 capsules once daily) because of the following reasons. Firstly, since the active ingredients in DJW and their pharmacokinetics are still unknown, we assumed that a multiple dosage regimen with shorter dosing interval should cause less fluctuation in plasma concentrations of active ingredients at steady state, leading to less fluctuation in therapeutic response (if there is a correlation between plasma concentration and therapeutic response). Secondly, since there is no evidence whether the

active ingredients of DJW exert their therapeutic effect via an inhibition of COX pathway similar to NSAIDs, taking DJW immediately after each meals (identical to most NSAIDs) is therefore a reasonable approach to avoid GI irritation (if any). Thirdly, prescribing DJW or diclofenac in the same manner (3 times a day) might be more convenient and practical as well as more successfully blinded the patients.

For a core set of outcome measures for phase III clinical trails in OA of the knee, the consensus development at OMERACT III [86] recommended that the following 4 domains should be evaluated: pain, physical function, patient's global assessment, and joint imaging (for studies of one year or longer). In our short-term study, we measured pain by using VAS assessing walking pain, standing pain, pain during climbing up and down the stairs, night pain, resting pain and total pain (similar to pain index of the Western Ontario and McMaster Universities Osteoarthritis Index, WOMAC). For the evaluation of physical function, we considered Lequesne's functional index, a questionnaire suggested by European League of Association for Rheumatology, instead of WOMAC disability index because WOMAC questionnaire assessing physical function seems not to be suitable for Thai patients whose daily activities are quite different from western life. For the evaluation of patient's global assessment, we used VAS to evaluate patient's overall opinion of improvement. Besides these parameters, VAS assessing pain during the most painful knee movement, stiffness (similar to WOMAC stiffness index), physician's overall opinion of improvement, and time for climbing up the stairs were also used for global judgement of effectiveness.

In both an ITT analysis and analysis on completers, pain scores, stiffness scores, Lequesne's functional index and time for climbing up the stairs at each time point decreased significantly when compared to their own base-line values. These data indicate that both DJW and diclofenac are effective in symptomatic treatment of OA of knee. However, the mean changes in some variables between the two groups were significantly different at the first few weeks after initiation of the treatment, but became indifferent afterwards. These differences suggest that the onset of DJW is significantly slower than diclofenac for at least 2 weeks (walking pain, standing pain, morning stiffness, stiffness after rest and total stiffness) or 3 weeks (Lequesne's functional index). Nonetheless, the effectiveness of both treatments was considered to be comparable after that. The reason why DJW needs a few weeks to exert its effect may be

due to 2 possibilities. Firstly, from the pharmacokinetic point of view, the elimination half-life of the active ingredients in DJW might be so long, it therefore needs weeks to accumulate until steady state concentration is reached (normally 4-5 times of half-life) and its maximal therapeutic effect is evident. Secondly, from the pharmacodynamic point of view, DJW may exert its effects via several probable mechanisms (similar to many novel biologic treatment of arthropathy) involved modifications of cartilage metabolism, normalized viscosity and elasticity of synovial fluid, etc. These mechanisms of action might resemble many chondroprotective drugs or symptomatic slow acting drugs in osteoarthritis (SYSADOA) such as glucosamine sulfate, intra-artricular hyaluronan, and others. These interventions always need a period of time to exert their therapeutic action. In this study, 3 patients with severe symptom in DJW group withdrew from the study due to ineffectiveness. All of them withdrew within 2 weeks after the treatment started, the time that the therapeutic effect of DJW had not yet set forth because of its delayed onset of action. Thus, in the future study or in medical practice, rescue analgesics (e.g., paracetamol as needed) should be recommended, especially in the first 2-3 weeks after initiation of DJW.

In the 1 and 2 months follow up period after the end of treatment, the number of remaining responders in DJW group was significantly more than that in diclofenac group. This result suggests that the duration of action of DJW is much longer than diclofenac which is in line with either its proposed longer elimination half-life or carry-over effect from chrodroprotection mentioned above or both.

The mean changes in total pain (500 mm) at week 4 compared to week 0 (week 0-4) in diclofenac and DJW group were –208.33 and –198.61 mm, respectively. After transformed data by using total score of 100 mm, the mean changes were -41.67 and -39.72 mm, respectively, which were greater than the value of –32.99 mm reported in diclofenac group from our previous study [87]. In addition, the mean changes in Lequesne's functional index in diclofenac and DJW group (-6.16 and –5.29, respectively) were also greater than the value of –4.80 in diclofenac group from our previous study [87]. In the present study, the reduction in VAS assessing pain (and stiffness) seemed to be higher than the reduction in Lequesne's functional index. This might be due to the different scales of both instruments. VAS is a continuous scale, whereas, Lequesne's functional index is an ordinal scale. Thus, if there was a significant reduction from

severe pain to mild pain, the Lequesne's functional index would change only minimally because mild pain meant that the pain is still existed.

The mean changes in body weight at each time point compared to the base-line values in diclofenac group were significantly different from DJW group. These might be due to the effect of diclofenac in inhibition of renal COX enzyme (especially COX-1), leading to salt-water retention [88]. In addition, even though concomitant antihypertensive treatment in both groups were not statistically different, the mean changes in systolic and diastolic blood pressure in diclofenac group tended to be less than DJW group. This less reduction in blood pressure might be partly the result from salt-water retention (increased intravascular volume led to increased cardiac output, and hence blood pressure). However, tendency of greater reduction in blood pressure as well as lack of weight gain during DJW treatment might be due to these possibilities. Firstly, therapeutic effect of DJW might not be substantially mediated via COX-1 inhibition, leading to less salt-water retention as supported by the data from our present animal studies. Secondly, some herbs might possess antihypertensive effect, leading to greater reduction in blood pressure when compared to diclofenac [23,31,45]. Nonetheless, the effects on blood pressure and salt-water retention should be further investigated.

This study demonstrated that DJW was safe and free from serious adverse effects. However, the gastrointestinal adverse effects in diclofenac group were quite low when compared to other short-term NSAIDs studies [89-90]. This might be due to exclusion of patients with high risk to adverse effects from NSAIDs during screening visit.