MATERIALS AND METHODS

ANIMAL STUDIES

A. Experimental animals

Male Sprague-Dawley rats weighing 100-120 g and 180-200 g as well as male Swiss albino mice weighing 20-40 g were purchased from the National Laboratory Animal Center, Nakorn Pathom. All animals were kept in a room maintained under environmentally controlled conditions of 24 ± 1 ^{0}C and 12 h light-dark cycle. The animals had free access to water and food and were acclimatized at least one week before starting the experiments.

B. Preparation of the ethanol extract of DJW

The schemes of crude drugs and their diagnostic structures are presented in Appendix 1. The proportion of fine powder of the raw materials used as ingredients (described in Appendix 2) were mixed thoroughly. The mixture was then macerated in 95% ethanol, allowed to stand for 24 h and was filtered through a filter paper using a vacuum pump. The maceration was repeated 2 times. All the filtrates were pooled and evaporated under reduced pressure and controlled temperature (50-60 ^{0}C) by using a vacuum rotary evaporator. The extract was then lyophilized and the yield of this extract was used in all experiments by suspending in 5% tween 80.

C. Preparation of the reference drugs

All reference drugs were suspended in 5% tween 80.

D. Drug administration

All reference drugs and the extract were orally administered in an equivalent volume of 0.5 ml/100 g body weight of the animal. Control groups received the same volume of vehicle by the same route.

E. Experimental protocol

1. Anti-inflammatory study

1.1 Carrageenin-induced hind paw edema in rats [57]

This method was used for investigation of the inhibitory effect of anti-inflammatory drugs on the edema formation induced by carrageenin.

Male rats of 100-120 g body weight were divided into 3 groups, 6 animals per group. A very high dose of DJW extract (8,000mg/kg), a 10 mg/kg dose of diclofenac or only vehicle (5% tween 80 in control group) were orally given 1 h prior to carrageenin injection. Lambda carrageenin was prepared as 1% suspension in sterile NSS. A volume of 0.05 ml of 1% carrageenin was injected intradermally into the plantar side of the right hind paw of an unanesthetized rat, which was restrained in a plastic cage.

Foot volume of animal was determined by mean of a volume displacement technique using a plethysmometer (model 7150, Ugo Basile, Italy). The right hind paw was immersed into the measuring chamber containing 0.05% NaCl in distilled water, exactly to an ink mark at anatomical hair line. Each paw volume was obtained from the average of 3 readings. The paw volume was measured prior to and at 1, 3 and 5 h after carrageenin injection.

The edema volume of the paw and the percent edema inhibition of each test compound were obtained by the following calculation:

$$EVx = PVx - PVo$$

$$\%EIx = \frac{\text{EVx of control group} - \text{EVx of test group}}{\text{EVx of control group}} \times 100$$

Where,

EVx = edema volume (ml) at time x

PVx = paw volume (ml) at time x

PVo = paw volume (ml) measured before carrageenin injection

%EIx = percent edema inhibition of test compound at time x

1.2 Cotton pellet-induced granuloma formation in rats [58]

This method was performed for investigation of the ability of an agent to inhibit the proliferative component of the subchronic and chronic inflammatory processes. It was slightly modified as follows:

Adsorbent cotton wool was cut into pieces weighing 20 ± 1 mg and made into a pellet. The pellets were then sterilized in a hot air oven (model 25, Arthur H. Thomas Co., USA) at 120 ^{0}C for 2 h. Male rats of 180-200 g body weight were divided into 4 groups, 6 rats per group. The first group was a control group that received 5% tween 80 only. The second and the third groups received a 5 mg/kg dose of prednisolone and a 10 mg/kg dose of diclofenac, respectively. The last group received a very high dose of DJW extract (8,000 mg/kg). Two pellets were implanted subcutaneously, one on each side of the abdomen of the animal under light ether anesthesia and sterile technique. The suture was then made and the animal was allowed to recover. All groups were administered orally in a once daily dosage regimen throughout the experimental period of 7 days. On the eighth day after cotton implantation, rats were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). After opening the thoracic cavity, heart blood was collected and centrifuged at 1000 rpm for 5 min and the serum was separated. The rat was then sacrificed and the abdominal skin was opened. The implanted pellets were dissected out and carefully removed from the surrounding tissues and weighed immediately for the wet weight. The thymus was also dissected out. Both cotton pellets and thymuses were dried at 60 ^{0}C for 18 h and their dry weight were determined. The change in body weight from the first and the last day of experiment was also recorded.

The granuloma formation, transudative weight and the percent granuloma inhibition of the test compounds were calculated according to the following formulae:

$$\text{Transudative weight} = Wtw - Wtd$$

$$GW\ (\text{mg/mg cotton}) = \frac{Wtd - Wti}{Wti}$$

$$\%GI = \frac{\text{GW of control group} - \text{GW of test group}}{\text{GW of control group}} \times 100$$

Where,

Wtw = wet weight of granuloma pellet (mg)

Wtd = dry weight of granuloma pellet (mg)

Wti = initial dry weight of cotton pellet determined before implantation (mg)

GW = granuloma weight (mg)

%GI = percent granuloma inhibition

Samples of serum were sent to Chiang Mai RIA Co., Ltd. for determination of the amount of alkaline phosphatase and total protein. The enzyme activity was expressed as units of enzyme/mg of serum protein.

2. Analgesic study

The analgesic activity of DJW was tested and compared with reference drugs using the formalin test [59]. Briefly, male Swiss albino mice weighing 20-40 g were divided into 6 groups, 6 mice per group. Three doses of DJW extract (250, 1,000, 4,000 mg/kg), 10 mg/kg dose of diclofenac and 50 mg/kg dose of codeine were given orally. The control group received 5% tween 80.

In the early phase assessment, 20 µl of 1% formalin in normal saline solution (NSS) was injected subcutaneously into the right dorsal hind paw of the mouse 1 h after reference drug or the extract. Then between 0-5 min after formalin injection, the time in seconds the mice spent for intensive licking the right dorsal hind paw were recorded.

In the late phase assessment, another set of 6 groups of mice was used. The formalin was injected 40 min after drug treatment and the licking time was recorded between 20-30 min after formalin injection.

Percent inhibition of licking response was calculated according to the following formula:

$$\% \text{ Inhibition of licking response} = \frac{\text{LT of control group} - \text{LT of test group}}{\text{LT of control group}} \times 100$$

Where,

LT = licking time

F. Statistical analysis

The data from the experiments were expressed as mean±SD. Statistical comparison between groups were analyzed by using one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test. p values less than 0.05 were considered significant.

CLINICAL TRIAL

A. Study design

This study was a randomized, double-blind, double-dummy, controlled trial. The 200 patients considered eligible for the study were randomized into 2 groups receiving DJW (DJW group) and diclofenac (diclofenac group), respectively. Each group was treated according to the protocol shown in Table 1 and each patient was treated for 4 weeks. This study was approved by the Medical Ethics Committee of the Faculty of Medicine, Chiang Mai University and was in compliance with the Helsinki Declaration.

Table 1. Treatment in DJW and diclofenac groups.

Treatment	DJW group	Diclofenac group
Capsule	Placebo*	Diclofenac
Herbal capsule	DJW	Placebo*

*Placebo was the dosage form of identical appearance containing inactive ingredients.

B. Patient selection

1. Inclusion criteria

Out-patients of either sex, age over 25, suffering from OA of knee (unilateral or bilateral) according to the criteria of the American College of Rheumatology [3] as: knee pain and radiographic osteophytes and at least 1 of the following 3 items: age over 50 years, morning stiffness not more than 30 min in duration, or crepitus on motion. The symptoms had to present for more than 3 months. After stop using usual medications for 7 days, visual analog scale (VAS) score assessing pain during the most painful knee movement had to be more than 40 and Lequesne's functional index [60] had to be more than 7 points (Appendix 3). Participants had to

be capable of walking. All patients had to sign the written consent form to participate in the study.

2. Exclusion criteria

The patients had to free from the following diseases or conditions: an underlying inflammatory arthropathy, hyperuricemia, expectation of surgery in the near future, recent injury in the area affected by OA of knee, intra-articular corticosteroid injections within the last 3 months, hypersensitivity to NSAIDs, abnormal liver or kidney function tests, major abnormal finding on complete blood count, history of coagulopathies, history of peptic ulceration and upper GI hemorrhage, uncontrolled hypertension, congestive heart failure, hyperkalemia, pregnancy, lactation and malignant tumors.

C. Treatment procedures

1. Diclofenac and its placebo

Twenty five mg film-coated tablets of commercially marketed diclofenac sodium (Voltaren®) were provided by Novartis (Thailand) Co., Ltd. In order to completely blind the patients, each diclofenac tablet was packed into capsule with the identical appearance of its placebo. Either diclofenac or placebo was prescribed 1 capsule, 3 times a day, immediately after meals.

2. DJW and its placebo

DJW and its placebo were prepared by Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University. The ingredients and preparation of DJW were listed in the Appendix 3. DJW was prescribed 3 g (6 capsules) each time, 3 times a day, immediately after meals. Its placebo in the identical appearance was made from cane sugar and prescribed the same dosage regimen as DJW.

D. Quality control and standardization of DJW

All raw materials used as ingredients in DJW were imported from the People's Republic of China. Xixin, Niuxi, Shudihuang and Rougui were imported from Shantou Traditional

Chinese Medicine Factory. The others were imported from Qixin Co., Ltd. (Hebei Province). The quality control and standardization of DJW (e.g., assessment of weight variation, disintegration time, screening for microorganisms and aflatoxin, etc.) were conducted by using guidelines recommended by the Food and Drug Administration of Thailand [61].

Although single preparation of DJW at the beginning of the study was theoretically required in order to minimize the variation in amount of active chemical substances prepared from different lots, this rationale seemed to be impractical in this study because very large amount of raw materials were needed in preparation of more than 25,000 capsules. Therefore DJW and its placebo were prepared in 4 separate lots. The quality control of DJW as well as screening for microorganism and aflatoxin in both DJW and its placebo were performed and needed to be passed before prescription of each lot. Every lot was used within 8 weeks in order to ascertain the stability of active substances and to avoid contamination of microorganism and aflatoxin during the study.

1. Screening method for microorganism contamination in DJW [62-65]

Three samples (1 g/sample) of herbal capsules and placebo from each lot were sampling. Each sample was suspended in 10 ml of 0.1% peptone water, pH 7.0. The suspension was diluted into serial ten fold dilutions from 1:10, 1:100, 1:1000, 1:10000 and 0.1 ml of the final dilution was cultured in the following media:

a) Trypticase soy agar for detecting total aerobic bacteria

b) Mannitol salt agar for detecting Staphylococcus aureus

c) Salmonella-Shigella agar for detecting Salmonella and Shigella

d) Alkaline peptone water and Thiosulfate-citrate-bile salt agar for detecting Vibrio spp.

e) Lactose broth, Brilliant Green Bile broth and MacConkey agar for detecting total coliforms and faecal coliform

f) Sabouraud dextrose agar for detecting aerobic fungi.

All of the cultured media were incubated at 37 ^{0}C for 24-48 h. The growing colonies, if any, were counted and identified for the species depending on the following characteristics and biochemical reactions.

a) Gram stain

b) Coagulase test

c) Catalase test

d) MR test

e) Voges-Proskrauer test

f) Triple Sugar Iron agar

g) Motility test

h) Indole production

i) Lysine decarboxylation

j) Urea

k) Citrate utilization

l) Sugar fermentation

2. Screening method for aflatoxin B1 contamination in DJW [66]

2.1 Sample preparation and extraction for Enzyme-Linked Immunosorbent Assay (ELISA)

The fine powder of all herbs from each lot was mixed together and sampling for three samples, 5 g/sample. Each sample was extracted by adding to 25 ml of 70% methanol and shaking vigorously for 3 min. The extract was filtered by pouring through a Whatman # 1 filter and the filtrate was stored at 2-8 ^{0}C until used for ELISA.

2.2 Direct competitive ELISA

One mixing well for each sample and each well for 4 controls (various concentrations of standard alfatoxin) and an equal number of antibody-coated wells were placed in the well holder. Prior to performing the test, all commercial reagents were allowed to reach room temperature (18-30 ^{0}C) and mixed well by swirling the reagent bottles prior to use. One hundred microliters of enzyme-labeled aflatoxin was placed in each mixing well. Then 100 µl of controls and samples were transferred to the mixing wells. The liquid in each well was mixed by pipetting up and down 3 times. One hundred microliters of the liquid were then transferred to the antibody-coated wells. The wells were mixed by sliding the well holder back and forth on a flat surface for 10-20 sec. After incubation at room temperature for 2 min, the contents of the antibody wells were

discarded. The distilled water was added to each well, and dumped from the wells and hit against absorbent paper. This step was repeated 5 times. One hundred microliters of substrate were then added to each well and mixed by sliding back and forth on a flat surface for 10-20 sec. After incubation for 3 min, 100 μl of stop solution was added to each well and mixed by sliding back and forth on a flat surface. The wells were read within 20 min after the addition of stop solution, in an ELISA reader using a 650 nm filter. The control optical densities were used to form the standard curve and the sample optical densities were plotted against the curve to calculate the exact concentration of the toxin.

E. Protocol outline

During the run-in period of 1 week (week 0), patients considered eligible for the study were informed to discontinue all analgesics, anti-inflammatory drugs, and other modalities for treatments of arthralgia and arthritis. At the beginning of week 1, patients who still met the eligibility criteria were randomized into 2 groups and treated for 4 weeks (Table 1). Research plan, treatment procedures and assessment during the study were demonstrated in Table 2. Other medications and treatment modalities for OA were prohibited during the study. In addition, a count of unused drug and placebo were made weekly in order to check compliance.

Table 2. Research plan and treatment procedures during the study.

Treatment groups	Week 0	Week 1	Week 2	Week 3	Week 4
1. DJW group	Run-in period	• DJW, orally, 3 g (6 capsules) each time, 3 times a day, immediately after meals *plus* • Placebo of diclofenac, orally, 1 capsule each time, 3 times a day, immediately after meals.			
2. Diclofenac group		• Placebo of DJW, orally, 6 capsules each time, 3 times a day, immediately after meals *plus* • Diclofenac (25 mg), orally, 1 capsule each time, 3 times a day, immediately after meals			
	Discontinuation of analgesics, anti-inflammatory drugs, and others modalities for treatments of arthralgia & arthritis.				
	↑ Screening Blood check	↑ Randomization Base-line assessment	↑ Assessment	↑ Assessment	↑ Assessment	↑ Assessment

F. Efficacy assessments

Clinical assessments were evaluated weekly and included the followings:

1. A horizontal 100-mm VAS.

a) A horizontal 100-mm VAS assessing walking pain, standing pain, pain during climbing up and down the stairs, night pain, resting pain, total pain, pain during the most painful knee movement, morning stiffness, stiffness after rest and total stiffness (assessed by the patient). The VAS ranged from 0 (no pain) to 100 (unbearable pain).

b) A horizontal 100-mm VAS assessing physician's and patients' overall opinions of improvement. The VAS ranged from 0 (no improvement) to 100 (best possible improvement). The assessment forms were designed so that the patient and evaluator could view their own previously recorded scores, but patient and evaluator were not allowed to view each other's VAS.

2. Lequesne's functional index, a questionnaire concerning the patient's daily activities (score ranging from 0-24) [60].

3. Time for climbing up the 10-step stairs.

4. The number of responders who exhibited, at the end of the treatment, a score of 0-30 mm on the 100-mm VAS assessing pain during the most painful knee movement or a score of 70-100 mm for the outcome measurement of patient's overall opinions of improvement.

5. The number of remaining responders (at 1 and 2 months after treatment) who met the following criteria: (1) Lequesne's functional index score less than that of week 0, (2) the patient's overall opinion of improvement was rated as better or much better than that of baseline, and (3) did not take any medications for OA except paracetamol tablets less than 14 tablets/week.

G. Safety assessment

Complete physical examination and non-directive questioning for adverse events were performed weekly for safety assessment during the study. The patient was asked to report to the principal investigator whenever serious adverse effects arose.

H. Statistical analysis

1. Statistical analysis within group

The mean VAS and Lequesne's functional index index between base-line and at the following weeks were compared by a non-parametric Wilcoxon's signed-rank test, whereas, the averaged time for climbing up the 10-step stairs was compared by a paired t-test.

2. Statistical analysis between the two groups

A non-parametric Wilcoxon's rank-sum test was used to determine whether the two groups differed in the physician's and patients' overall opinions of improvement. In addition, the mean changes in the VAS assessing pain and stiffness, as well as Lequesne's functional index were compared by a non-parametric Wilcoxon's rank-sum test. A student's t-test was used to compare the mean changes in time for climbing up the 10-step stairs. The number of responders or remaining responders, and the number of patients experienced adverse events were evaluated by chi-square or Fisher's exact test.