MATERIALS AND METHODS

1. Plant material

Stems of *Diospyros variegata* Kruz. were collected in Chiangmai, Thailand. Voucher specimen of the plant (QBG 6742) was deposited at the Herbarium of the Queen Sirikit Botanical Garden, Chiangmai, Thailand.

2. Preparation of the extract and fractions

One kilogram of dried stem was ground and macerated with methanol at room temperature overnight. The maceration was repeated three times and then filtered. The crude methanol extract was evaporated under reduced pressure at a temperature of 55°C. The thick residue obtained (64.68 g) seemed to separate into two layers. The top layer was oily liquid with black colour. The bottom layer was a thick residue.

The crude methanol extract was then partitioned with hexane in equal volume for three times. The hexane fraction was pooled and evaporated under reduced pressure at a temperature of 45°C, yielding 15.74 g of hexane fraction, which was 1.57% w/w of raw material and 24.34% w/w of crude methanol extract. The residue left after partitioning with hexane was designated the methanol fraction which yielded 41.28 g

after drying (4.13% w/w of raw material and 63.82% of crude methanol extract). The scheme of preparation of the hexane and methanol fractions is shown in Fig. 3

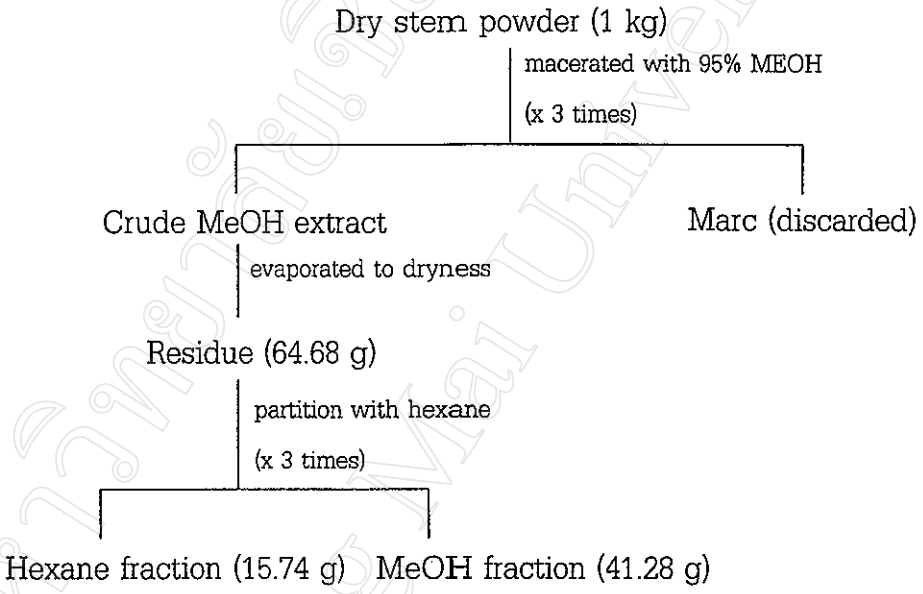

Fig. 3 Preparation of hexane and methanol fractions

3. Experimental animals

Adult Spraque-Dawley rats, weighing 40-60 g and 180-300 g and male Swiss-Albino mice, weighing 30-40 g, were purchased from the National Laboratory Animal Center, Salaya, Mahidol University, Nakorn Pathom, Thailand.

The animals were kept in animal rooms where the temperature was maintained at 24 ± 1°C with a 12 hour light-

dark cycle. The animals had free access to food and water and were acclimatized for at least one week before starting the experiments.

4. Drug administration

All test drugs were suspended in 5% Tween 80 and given intraperitoneally to the animals, except in the ear edema model in which test drugs were dissolved in acetone and applied topically. Animal in control groups received vehicle in the same volume and by the same route.

5. Analgesic test

The analgesic activity of test agents was tested and compared with reference drugs using three following methods.

5.1 Writhing response in mice

Male mice, weighing 30-40 g were used. The method was done essentially as described by Nakamura *et al.*, 1986

A typical "writhing response" was produced by an intraperitoneal injection of a 0.1ml/10g body weight of a 0.75% acetic acid into the mouse. This "writhing response" is characterized by intermittent contraction of abdomen, twisting and turning of the trunk followed by extension of the hind legs,

beginning 5 minutes after injection of acetic acid and persisting more than one hour. The test fractions, aspirin and morphine were tested for their ability to suppress "writhing response" by intraperitoneal administration 30 min before acetic acid injection. In control group, the mice received only vehicle.

After challenge, the mice were placed in a translucent plastic box and the number of writhes, starting 5 min after acetic acid injection was counted during continuous observation for 15 min. The percentage of protective effect was calculated.

5.2 Tail-flick test

Male rats, weighing 180-200 g, were used. The method used was that of D' Amour and Smith (1941) as modified by Gray *et al.*, (1970)

The rat was placed on the tail flick apparatus (SOCSEL, model DS 20, Ugo Basile, Italy), such that the tail occluded a slit over a photocell. Heat was applied by a 100 Watt lamp mounted in a reflector (Fig. 4). The apparatus was so arranged that when the operator turned on the lamp a timer was activated. When the rat felt pain and flicked its tails, the light was able to fall on the photocell and timer was automatically stopped. The voltage

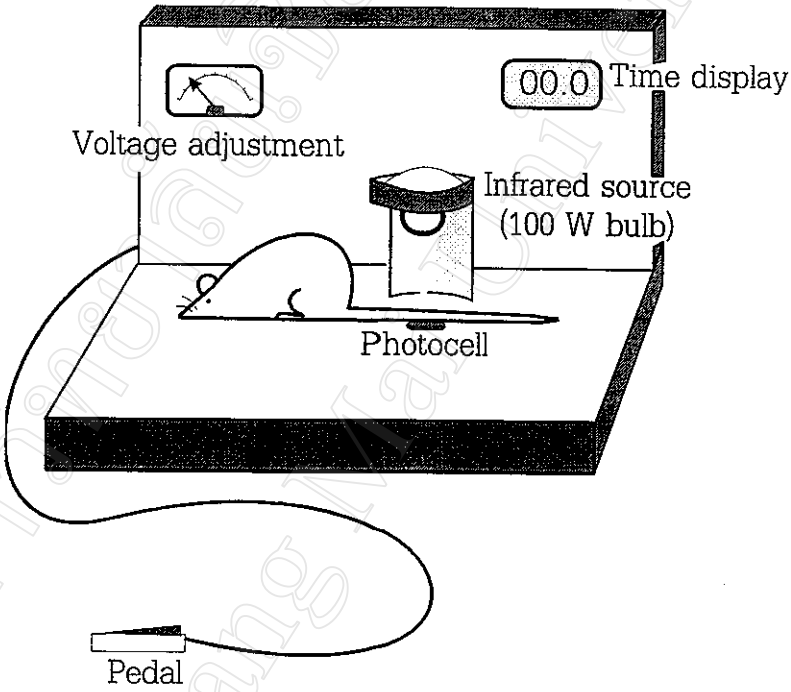

Fig. 4 Diagram illustrating the method for tail-flick test

was adjusted to give a normal reaction time of 2-4 s. The cut-off time for the stimulus i.e. the maximum time, which an unflicked tail can be exposed to the heat without damage, was 10 s. Two control readings, taken 30 min apart, were averaged and constituted the control reaction time.

Test fractions, aspirin and morphine were administered intraperitoneally 30 min before re-exposure to the noxious stimulus.

The analgesic response was calculated as a percentage of the maximum possible response time (Harris and Pierson, 1964) by the following equation:

$$\% \text{ maximum possible effect} = \frac{\text{test - control}}{\text{10 - control}} \times 100$$

where,

test = mean test reaction time (s)

control = mean control reaction time (s)

10 = cut-off time (s)

5.3 Formalin test

The formalin test comprised the early phase and the late phase assessment of the analgesic effect, which was performed separately according to the method of Hunskaar and Hole (1987). Male mice, weighing 30-40 g, were injected

intraperitoneally with either test fractions or aspirin or morphine.

In the early phase assessment, 20 μl of 1% formalin in normal saline solution (NSS) was injected subcutaneously into the right dorsal hindpaw of the mouse 30 min after the sample treatment. Then between 0-5 min after formalin injection, the time in seconds the mouse spent for intensive licking the right dorsal hind paw was determined (Fig. 5).

In the late phase assessment, another group of mice was used. The formalin was injected 10 min after test drug treatment and the licking time was determined between 0-10 min after formalin injection (Fig. 6).

6. Anti-inflammatory test

Ear edema-induced in rats by ethyl phenylpropiolate (EPP) and arachidonic acid (AA)

The methods of Brattsand *et al.*, (1982) and Young *et al.*, (1984) were used with slight modification. Male rats weighing 40-60 g were used. EPP and AA were dissolved in acetone.

Ear edema was induced by topical application of either EPP or AA to the inner and outer surfaces of both ears. Phenylbutazone and phenidone used as reference drugs as well as test fractions were dissolved in acetone and applied topically

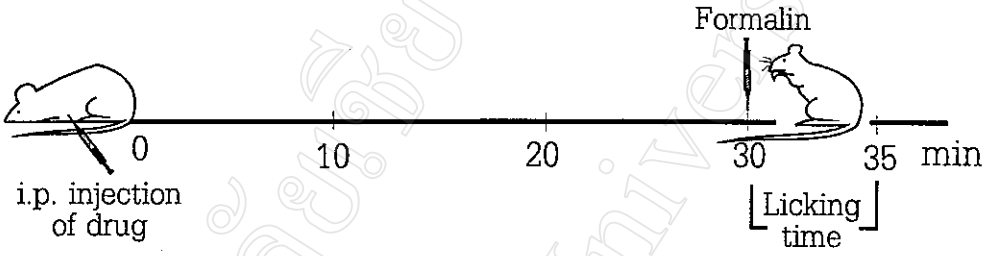

Fig. 5 Diagram illustrating the method for formalin test (early phase)

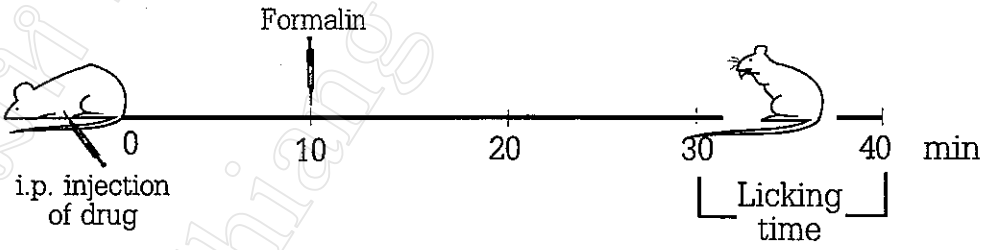

Fig. 6 Diagram illustrating the method for formalin test (late phase)

to the ear, just before the irritants. Before and at 15, 30, 60 and 120 min after edema induction, the thickness of each ear was measured with the vernier calipers. The increase in ear thickness was compared with the vehicle-treated group and the percent inhibition was calculated as follow:

$$ED_x = ET_x - ET_0$$

$$\% EDI = \frac{ED_t - ED_c}{ED_c} \times 100$$

ED_x = Edema thickness of time x

ET_x = Ear thickness at time x

ET_o = Ear thickness before EPP or AA application

ED_c = Edema thickness in control group

ED_t = Edema thickness in test group

EDI = Edema inhibition

7. Antipyretic test

The antipyretic activity of test fractions was tested and compared with aspirin, using the method described by Teotino *et al.*, (1963) as follows:

Male rats, weighing 280-300 g, were used. They were housed and maintained under uniform environmental

conditions. Disturbances likely to excite them were avoided. Before pyrexia was induced, the animals were restrained in plastic cages and the initial rectal temperatures were recorded using a ten channel electric thermometer (EXACON,model MC 8940, EXACON Scientific Instruments Aps, Denmark) connected with the probes (model H-RRA, EXACON Instruments Aps, Denmark) which were inserted into the rat rectums to about 5 cm depth (Fig. 7). In order to adapt the rats to the handling procedure for probe insertion basal rectal temperatures were taken 1 h after probe insertion. Thereafter hyperthermia was induced in rats by subcutaneous injection of 1 ml/100g body weight of 20% yeast in NSS. When the temperature was at a peak, 18 h after yeast injection, the rectal temperatures were again recorded.

Those animals that showed a rise in rectal temperature of more than 1°C were used. Test fractions and aspirin were then administered intraperitoneally and the rectal temperatures of animals were recorded at 30 min interval for two hours following drug treatment.

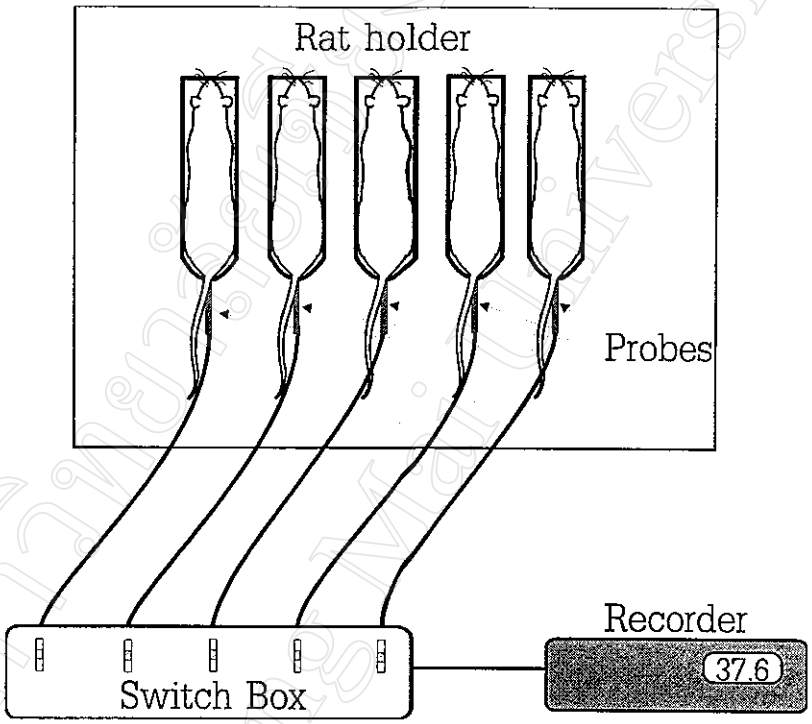

Fig. 7 Diagram illustrating the method for yeast-induced hyperthermia

8. Statistic analysis

All results are expressed as mean ± S.E.M. of six experimental (n = 6). Student's t-test was applied to the results to evaluate the significance of differences. The values exceeding 95% confidence limits were considered to be significant.

9. Drugs and chemicals

9.1 Drugs

a) Acetylsalicylic acid (aspirin, Vidhyasom Co., Ltd., Thailand)

b) Morphine (The Government Pharmaceutical Organization, Thailand)

c) Phenidone (Sigma Chemical Co; U.S.A)

d) Phenylbutazone (Sigma Chemical Co., U.S.A.)

9.2 Vehicles

5% Polysorbate 80 U.S.P. (Tween 80, Sigma Chemicals Co., U.S.A.)

Acetone (Merck KGaA, Damstadt, Germany)

9.3 Irritants

a) Brewers yeast (Sigma Chemicals Co., U.S.A.)

b) Arachidonic acid (AA) (Sigma Chemicals Co., U.S.A.)

c) Ethyl phenylpropiolate (EPP) (Aldrich Chemicals Co., U.S.A.)

d) Formalin 37% (The Government Pharmaceutical Organization, Thailand)

e) Glacial acetic acid B.P.C. 1973 (The Government Pharmaceutical Organization, Bangkok, Thailand)