MATERIALS AND METHODS

1. Preparation of the extracts

Leaf, twig, stem and bark of *O. integerrima*. Merr. were separately extracted by methanol. All extracts well provided from Prof. Dr. Vichai Reutrakul, Department of Chemistry, Faculty of Science, Mahidol University, Thailand, which are defined as;

1.1 OI-LF (methanol extract from leaf)

1.2 OI-TW (methanol extract from twig)

1.3 OI-ST (methanol extract from stem)

1.4 OI-BK (methanol extract from bark)

2. Experimental animals

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 $\pm$ 1 °C with approximately 45% relative humidity. They were expose to 12 h light-dark cycle. The animals had free access to food and water and were acclimatized for at least one week before starting the experiments.

3. Drug administration

The Ochna extracts and reference drugs were suspended in 10% acacia and given intraperitoneally to the animals, except in the ear edema model in which all test substances were dissolved in absolute ethanol and applied topically. Animals in control groups received vehicle in the same volume and by the same route.

4. Analgesic test

The Ochna extracts and reference drugs were tested and compared in analgesic activity using three following methods.

4.1 Writhing response

Male albino 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 0.1 ml/10 g 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 min after injection of acetic acid and persisting more than 1 h. The Ochna extracts, 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 inhibition was calculated by comparing with controls according to the following formula:

$$\% \text{ Inhibition} = \frac{W_c - W_t}{W_c} \times 100$$

Where W_c and W_t are mean number of writhes in control and treated animals, respectively.

4.2 Tail-flick test

The male albino mice weighing 20-40 g were used. The method was done essentially as described by Janssen, 1963. The Ochna extracts, aspirin and morphine were administered intraperitoneally 30 min before re-exposure to the noxious stimulus.

The basal reaction time of each mouse was determined using tail withdrawal response, with one-third of the tail immersed in a water bath at 55 °C. A cut off time of 30 s was fixed to avoid tissue damage. Reaction time was noted after every 30 min for a total period of 150 min.

4.3 Formalin test

The formalin test comprised the early phase and the late phase assessment of the analgesic effect that was performed separately according to the method of Hunskaar and Hole (1987). Male Swiss-Albino mice, weighing 30-40 g, were injected intraperitoneally with Ochna extracts, 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 0-10 min after formalin injection, the time in seconds the mice spent for intensive licking the right dorsal hindpaw was determined (Figure 2).

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 20-30 min after formalin injection (Figure 3).

5. 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) with slight modification were used. Male rats weighing 40-60 g were used. EPP and AA dissolved in ethanol. Ear edema was induced by

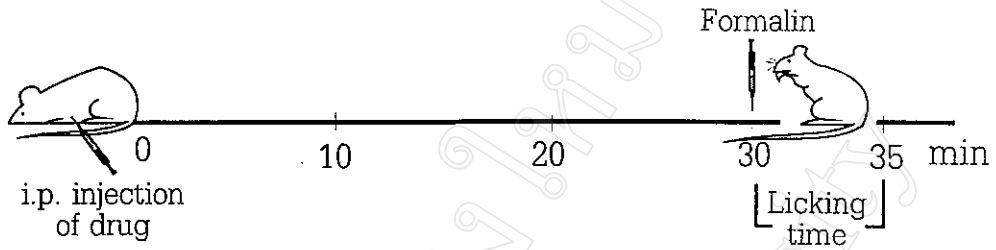

Figure 2. Diagram illustrating the method for formalin test (early phase) in mice

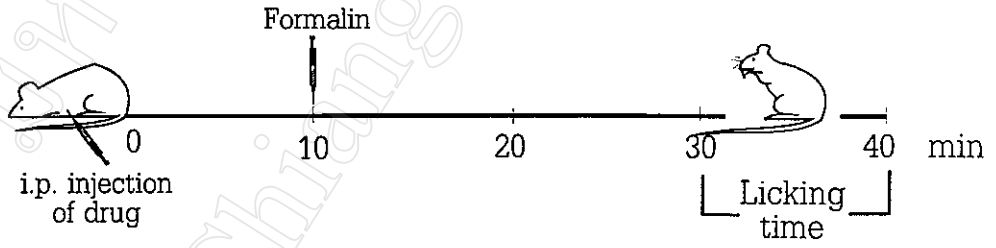

Figure 3. Diagram illustrating the method for formalin test (late phase) in mice

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 Ochna extracts were dissolved in absolute ethanol and applied topically to the ear, just before the irritants (EPP or AA). 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

$$ED_x = Et_x - Et_o$$

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

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

6. Antipyretic test

The antipyretic activity of Ochna extracts 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 Inturments 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 (Figure 4). In order to adapt the rats to the handling procedure for probe insertion, the basal rectal temperatures were taken 1 h after probe insertion. Thereafter hyperthermia was induced in rats by subcutaneous injection of 1 ml/100 g body weight of 20% brewers 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 extracts and aspirin were then administered intraperitoneally and the rectal temperatures of animals were recorded at 30 min interval for 2 h following drug treatment.

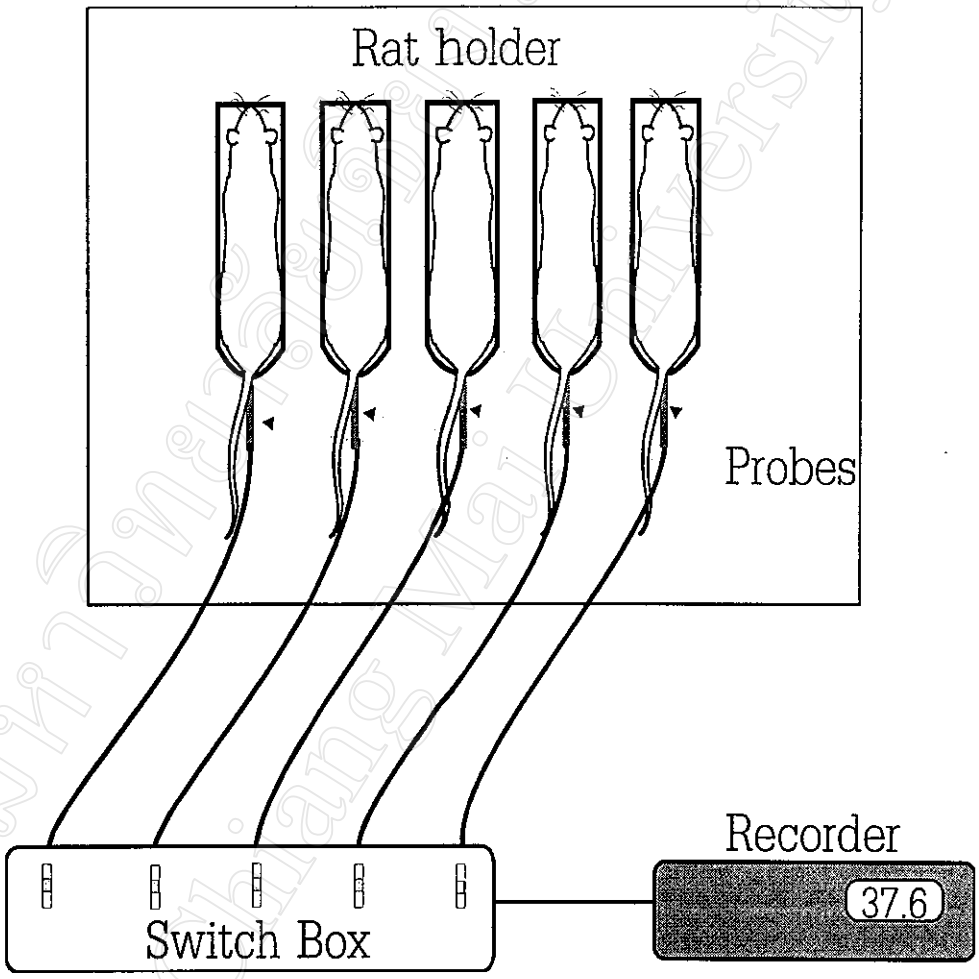

Figure 4. Diagram illustrating the method for yeast-induced hyperthermia

7. Toxicity test and LD_{50} determination

The LD_{50} was assumed by using 50% death within 72 h following intraperitoneally injection of various doses of the extract that showed highly analgesic and antipyretic activities. Male mice weighing 30-40 g were used in this experiment.

8. Statistic analysis

All results are expressed as mean ± S.E.M (n=6). Student's t-test and oneway ANOVA were 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

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

9.1.2 Morphine (The Government Pharmaceutical Organization, Thailand)

9.1.3 Phenidone (Sigma Chemical Co., U.S.A.)

9.2 Vehicle

9.2.1 acacia (Vidhyasom Co., Ltd, Thailand)

9.2.2 ethanol (Merck, Germany)

9.3 Irritants

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

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

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

9.3.4 Formalin 37% (The Government Phamaceutical Orginazation, Thailand)

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