CHAPTER 3

MATERIALS AND METHODS

Materials

1. Plant material

Kaempferia galanga L. (Proh Hom) was collected in January, 2006 from Chana District, Songkhla Province, Thailand. The plant was identified by Professor Puangpen Sirirugsa, Department of Biology, Faculty of Science, Prince of Songkla University. A voucher specimen NO. 200601 of plant material has been deposited in the PSU Herbarium, Department of Biology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The 20 kg of fresh plant of *K. galanga* was cleaned with tap water and air-dried at room temperature. The dried plants were pulverized by an electric blender to give 5 kg of coarsely powder and stored in airtight containers.

2. Extraction procedure

The coarsely powder of *K. galanga* (5 kg) was macerated with 20 L of methanol for 7 days at room temperature. This extraction process was repeated 2 times and filtered. The combined filtrate was filtered through Buchner funnel suction with Whatman No. 40 filter paper and evaporated by rotary evaporator at 40-60 °C and lyophilized by freeze-dried to give a total solid residue of 119.2 g (yield 0.60 %w/w) which was stored in a closed bottle and kept in a refrigerator at temperature below 4 °C until testing. Before testing of the extract for the pharmacological activities, the extract at different concentrations was prepared by dissolving in cosolvent (Propylene glycol : Tween 80 : Distilled water at the ratio 1:4:5, respectively).

3. Animals

Male albino mice with weighing ranging from 28-40 g were used for analgesic activity testing for hot plate, writhing, and formalin tests. Male Wistar rats weighing ranging 140-230 g were used for analgesic activity testing method for tail flick test; antipyretic and anti-inflammatory activities. All animals were kept in the room maintained environmentally controlled under conditions of temperature (26ºc) and 12 :12 h light/dark cycle. Food and water were given

ad libitum unless otherwise specified. All animals used in this study were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai, Songkhla, Thailand. All procedures described were reviewed and approved by the Institutional Committee for Ethical Use of Animals, Prince of Songkla University, Hat Yai, Thailand (No.ศธ 0521.11/426).

4. Chemicals and Instruments

The drugs were used as following; morphine sulfate (Sigma), naloxone hydrochloride dihydrate (Sigma), brewer's yeast (Sigma), carrageenan (Sigma), acetylsalicylic acid or aspirin (Sigma), pentobarbital (Sigma), propylene glycol (Sigma), tween 80 (Sigma), acetylcholine (sigma), acetic acid and 40% formalin (A.R. grade, J.T., Baker) and methanol (Commercial grade).

The instruments used included the tail flick apparatus (Socrel model DS20), Plethysmometer (Ugo basile, Varese, Italy), hot plate and digital thermometer (9SK-1250 MC, Sato Keiryoki mfg Co., Ltd., Japan).

5. Drugs and extract administration

The cosolvent, extract and reference drug aspirin were administered by oral gavage. Animals were divide into 5-8 groups. Each group comprised of 10 animals of mice or rats for analgesic and antipyretic testing, and eight animals in anti-inflammatory study.

Group	Name group	Detail	Test
1	Control	Cosolvent composed of Tween 80 : Propylene glycol : Distilled water at the ratio of 1:4:5 at doses of 10 ml/kg (mice) and 5 ml/kg (rats).	Analgesic, anti-pyretic and anti-inflammatory
2, 3	Standard drug	1) Aspirin at the dose of 100 mg/kg, orally.	Writhing, formalin, anti-pyretic and inflammatory
		2) Morphine sulfate at the dose of 5 mg/kg, subcutaneously.	Formalin, hot plate and tail flick
		3) Naloxone at the dose of 2 mg/kg, Intraperitoneally	Hot plat and tail flick

Group	Name group	Detail	Test
4, 5, 6	MEKG	Methanol extract of *K. galanga* (MEKG) at doses of 50, 100 and 200 mg/kg, orally, respectively.1)	Analgesic, antipyretic and anti-inflammatory
7, 8	Antagonist effect	Naloxone at the dose of 2 mg/kg given intraperitoneally before morphine 5 mg/kg, subcutaneously 2) Naloxone at the dose of 2 mg/kg given intraperitoneally before MEKG at the dose of 200 mg/kg	Hot plate and tail flick

Methods

1. Acute toxicity

The up-and-down method described by Bruce (1985) was used in this study. This method used for acute toxicity testing has been developed and statistically evaluated and permits a reduction in the number of animal used. In the procedure, animals are dosed one at time. If an animal survives, the dose for the next increased, if it died the dose is decreased and observe each animal for 1 or 2 days before dosing the next animal. The first dose was begin at 300 mg/kg and adjusted by a constant multiplicative factor, viz. 1.5 up to 5 g/kg for the experiment. The methanol extract of *K. galanga* was orally administered to a group of mice and rat both male and female. Behavior parameters were observed during a period of 8 h and 7 days after administration as convulsion, hyperactivity, sedation, grooming, loss of righting reflex, increased or decreased respiration. Food and water was given *ad libitum.*

2. Antinociceptive activity

2.1. Writhing test

The method described by Koster *et al.* (1959). Male albino mice weighing 28-40 g were divided into 5 groups and each group consisted of 10 mice.

Group 1 : Control (cosolvent 10 ml/kg, p.o.).

Group 2 : Aspirin (100 mg/kg, p.o.)

Group 3 : MEKG 50 (mg/kg, p.o)

Group 4 : MEKG 100 (mg/kg, p.o)

Group 5 : MEKG 200 (mg/kg, p.o)

The control group (group 1) received cosolvent (10 ml/kg, p.o.). Group 2 received a reference drug aspirin at the dose of 100 mg/kg, p.o. Group 3, 4 and 5 received the extract at doses of 50, 100 and 200 mg/kg, p.o., respectively. After 30 min of treatment, each mouse of each group was administered intraperitoneally with 0.6%(v/v) acetic acid in 0.9% normal saline at dose of 10 ml/kg. The mice were then observed for the number of abdominal constriction and stretching and counted over a period of 0-20 minute. The schematic plan of the writhing test was illustrated in Figure 15.

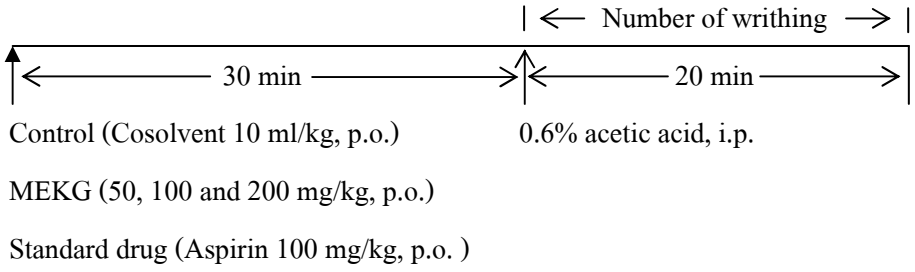


Figure 15. Schematic plan of the writhing test

A reduction in the writhing number compared to control group was evaluated for analgesia which was expressed as % inhibition of writhings. The percentage of inhibition was determined for each experimental group as following formula.

$$\text{Inhibition (\%)} = \frac{\text{No. of writhing(control group)} - \text{No. of writhing(experimental group)} \times 100}{\text{No. of writhing (control group)}}$$

2.2. Formalin test

The formalin test was performed according to the method of Hunskarr *et al.* (1985). Male albino mice (weighing 28-40 g) were divided in to 6 groups of 10 mice each.

Group 1 : Control (cosolvent 10 ml/kg, p.o.).

Group 2 : Aspirin (100 mg/kg, p.o.)

Group 3 : Morphine sulphate (5 mg/kg, s.c.)

Group 4 : MEKG 50 (mg/kg, p.o)

Group 5 : MEKG 100 (mg/kg, p.o)

Group 6 : MEKG 200 (mg/kg, p.o)

The control group received cosolvent (10 ml/kg, p.o.). Group 2 and 3 received reference drug aspirin (100 mg/kg, p.o.) and morphine sulphate (5 mg/kg, s.c.), respectively. Group 4, 5 and 6 received the extract at doses of 50, 100 and 200 mg/kg, p.o. After 30 min of treatment (except 15 min for morphine), 20 µL of 2.5% formalin in 0.9% normal saline was injected subcutaneously to a hind paw of each mouse. The time spent in licking of the injected paw was recorded. The data were expressed as the total licking time in early phase (0-5 min) and late phase (15-30 min) after formalin injection. The schematic plan of the formalin test was illustrated in Figure 16.

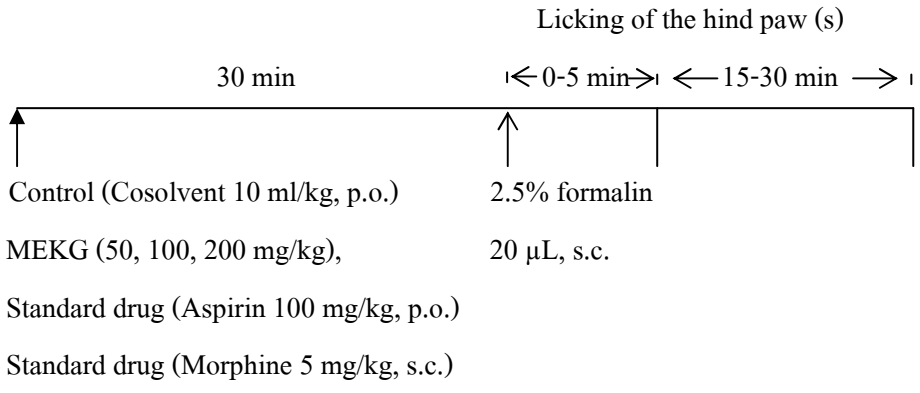


Figure 16. Schematic plan of the formalin test

The time spent licking the injected paw was evaluated for analgesia which was expressed as % inhibition of the time spent licking. The percentage of inhibition was determined for each experimental group as followsing formula.

$$\text{Inhibition (\%)} = \frac{\text{Control latency} - \text{Post-drug latency}}{\text{Control latency}} \times 100$$

2.3. Hot plate test

The hot plate test was carried out according to the method described by Woolfe and MacDonald (1944). Male albino mice weighting 28-40 g were divided in 8 groups of 10 mice each.

Group 1 : Control (cosolvent 10 ml/kg, p.o.).

Group 2 : Morphine sulphate (5 mg/kg, p.o.)

Group 3 : Naloxone (2 mg/kg, i.p.)

Group 4 : MEKG (50 mg/kg, p.o.)

Group 5 : MEKG (100 mg/kg, p.o.)

Group 6 : MEKG (200 mg/kg, p.o.)

Group 7 : Naloxone (2 mg/kg, i.p.)+ Morphine (5 mg/kg, s.c.)

Group 8 : Naloxone (2 mg/kg, i.p.)+ MEKG (200 mg/kg, p.o.)

The control group (group 1) received cosolvent (10 ml/kg, p.o.). Group 2 and 3 received standard drug morphine sulphate (5 mg/kg, p.o.) and naloxone (2 mg/kg, i.p.), respectively. Group 4, 5 and 6 received the extract at doses of 50, 100 and 200 mg/kg, p.o., respectively. Group 7 and 8 received naloxone (2 mg/kg, i.p.) 10 min before morphine (5 mg/kg, s.c.) or the extract at the dose of 200 mg/kg, p.o., respectively. After 30 min of treatment with each extract dose or cosolvent (except 15 min for morphine and 10 min for naloxone), mice were placed on a hot plate maintained at 55 °C ± 1 °C. Latency of nociceptive reponses such as licking, flicking of a hind limb or jumping was measured, the nociceptive response was measured every 15 min over a 90 min period at intervals of 30, 45, 60 and 90 min. The cut-off time of

observation was 45 seconds. Only the mouse showed nociceptive responses within 15 seconds was used in the experiments**.** The schematic plan of the hot plate test was illustrated in Figure 17.

2.4. Tail flick test

The tail flick test described by D'Amour and Smith (1941) was used in this study. Male Wistar rats weighting 140-180 g were divided into 5 groups of 10 rats each.

Group 1 : Control (cosolvent 5 ml/kg, p.o.).

Group 2 : Morphine sulphate (5 mg/kg, i.p.)

Group 3 : Naloxone (2 mg/kg, i.p.)

Group 4 : MEKG (50 mg/kg, p.o.)

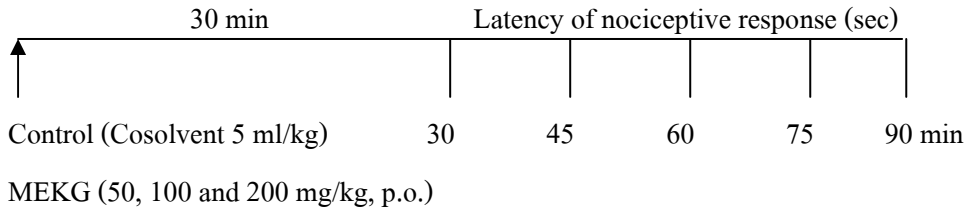
Group 5 : MEKG (100 mg/kg, p.o.)

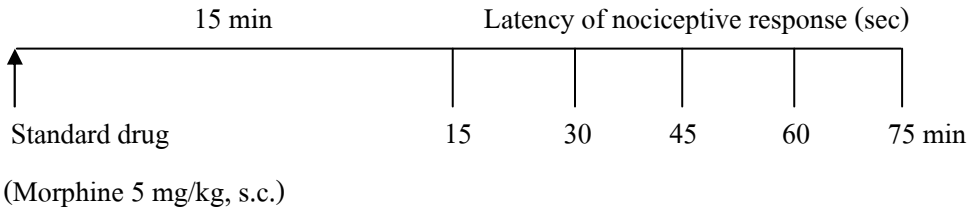
Group 6 : MEKG (200 mg/kg, p.o.)

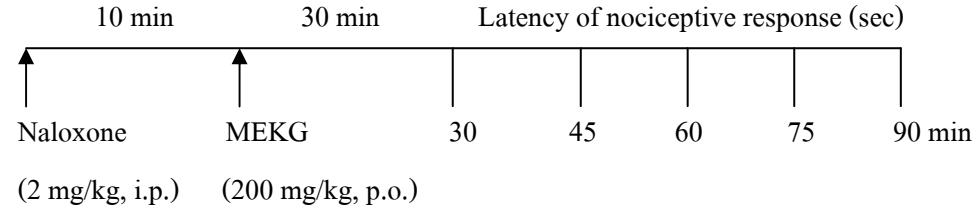
Group 7 : Naloxone (2 mg/kg, i.p.)+ Morphine (5 mg/kg, s.c.)

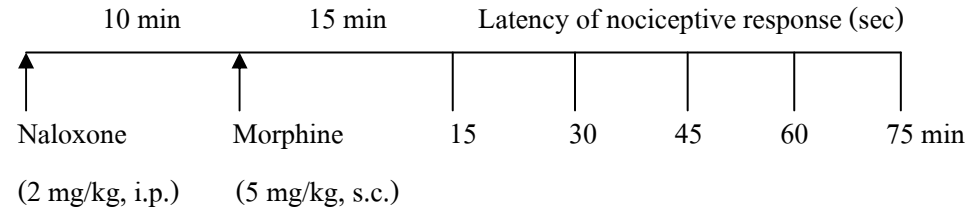
Group 8 : Naloxone (2 mg/kg, i.p.)+ MEKG 200 mg/kg, p.o.

The control group received cosolvent at 5 ml/kg, p.o. Group 2 and 3 received standard drug morphine sulphate (5 mg/kg, i.p.) and naloxone (2 mg/kg, i.p.), respectively. Group 4, 5 and 6 received the methanol extract of *K. galanga* at doses of 50, 100 and 200 mg/kg, p.o., respectively. In group 7 and 8, naloxone (2 mg/kg, i.p.) was given 10 min before morphine sulfate (5 mg/kg, i.p.) and MEKG (200 mg/kg, p.o.). After 15 min of morphine administration or 30 min of MEKG administration, the reaction time was determined. In all groups in this experiment, each rat was placed on the tail flick apparatus (Socrel model DS20, Ugo basile) and the tail flick response was measured by gently placing the rat tail on a center position of light beam. The reaction time was measured by focusing an intensity controlled beam of light on the distal one-third portion of the animal tail (about 4 centimeters from tip). The time taken by the animal to withdraw (flick) its tail from heat induced by the light beam was recorded as the reaction time. Only the rat that showed nociceptive response within 3 second was used for the experiments. A cut-off time was 10 second to prevent any injury to the rat tail. The nociceptive response was measure at 30, 45, 60, 75 and 90 min after administered agents. The schematic plan of the tail flick test was illustrated in Figure 17.









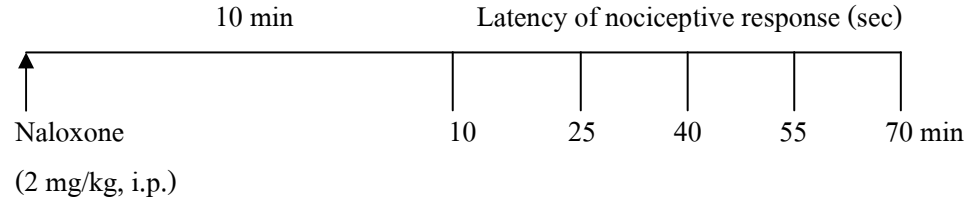


Figure 17. Schematic plan of the hot plate test and tail flick test

3. Antipyretic activity

Antipyretic activity was tested using the slightly modifying method described by Adam *et al.* (1968). Male Wistar rats were fasted overnight with water *ad libitum* before experiments. Pyrexia was induced by subcutaneous injection of 20% (w/v) brewer's yeast suspension (10 ml/kg) into animal's dorsum region. Nineteen hours after injection, the rectal temperature of each rat was measured using a digital thermometer. The probe was attached to a digital display and was inserted 2 cm into the rectum. Only rats that showed an increase in temperature of at least 0.7ºc were used for the experiments and the initial rectal temperature was recorded. After 30 min, male Wistar rats, weighing 140-180 g, were divided into 5 groups of 10 rats each. The control group received cosolvent at the dose of 5 ml/kg, p.o. The experimental groups was administered with the methanol extract of *K. galanga* at doses of 50, 100 and 200 mg/kg, p.o. The standard group aspirin was administered at the dose of 100 mg/kg, p.o.. Temperature was measured at 1, 2, 3, 4 and 5 h after each agent administration. The schematic plan of the brewer's yeast-induced pyrexia was illustrated in Figure 18.

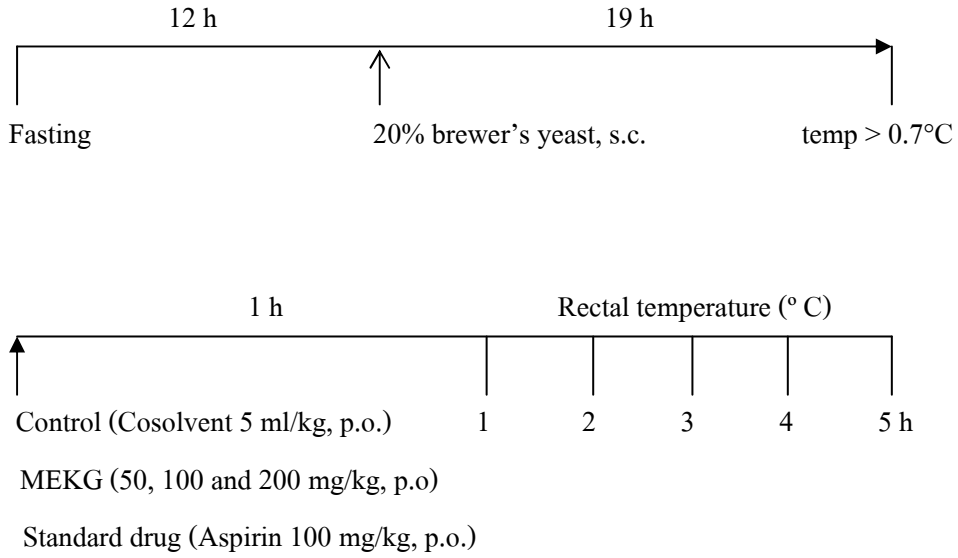


Figure 18. Schematic plan of the brewer's yeast-induced pyrexia

4. Anti-inflammatory activity

4.1. Acute inflammation (carrageenan-induced paw edema test)

This experiment was performed according to the method described by Winter *et al.* (1962). The initial right hind paw volume of the rats was measured using a Plethysmometer (Ugo basile). Male Wistar rats, weighing 140-180 g, were divided into 5 groups of 10 rats each.

Group 1 : Control (cosolvent 5 ml/kg, p.o.).

Group 2 : Aspirin (100 mg/kg, p.o.)

Group 3 : MEKG 50 (mg/kg, p.o)

Group 4 : MEKG 100 (mg/kg, p.o)

Group 5 : MEKG 200 (mg/kg, p.o)

The Control group (group 1) received cosolvent at the dose of 5 ml/kg, p.o. Group 2 received the standard drug aspirin at the dose of 100 mg/kg, p.o. Group 3, 4 and 5 received the methanol extract of *K. galanga* at doses of 50, 100 and 200 mg/kg, p.o., respectively. After 30 minutes, each rat of all groups was subcutaneously injected with 0.1 ml of 1 % (w/v) carrageenan in 0.9% normal saline into the subplantar region of the right hindpaw. The volume of right hind paw was measured at 0.5, 1, 2, 3, 4 and 5 h after carrageenan injection and edema volume was determined. The data were expressed as percentage of swelling compared with initial hind paw volume of each rat. The schematic plan of the carrageenan-induced paw edema experiment was illustrated in Figure 19.

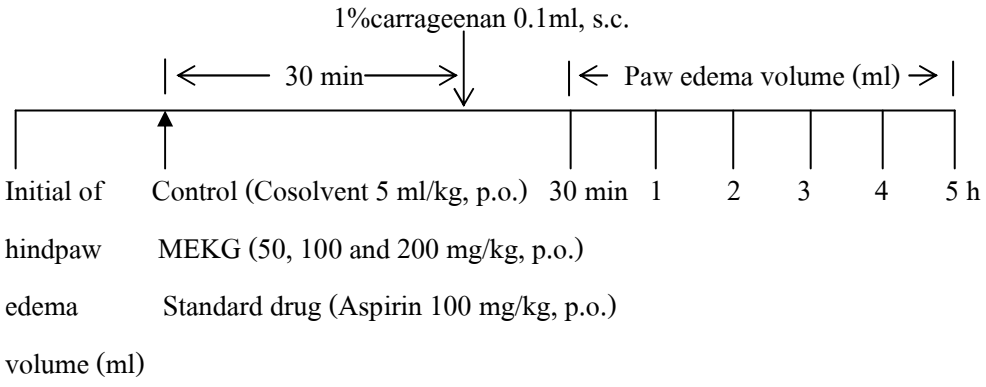


Figure 19. Schematic plan of the carrageenan-induced paw edema

The volume of hind paw was evaluated for anti-inflammatory activity and was expressed as % inhibition of the hind paw volume. The percentage of inhibition was determined for each experimental group as following formula.

$$\text{Inhibition (\%)} = \frac{(V_t\text{-}V_o)_{control} - (V_t\text{-}V_o)_{treated}}{(V_t\text{-}V_o)_{control}}$$

V_t = volume of hind paw after carrageenan injection

V_o = volume of hind paw before carrageenan injection

4.2. Chronic inflammation (cotton pellet granuloma test)

The rat-induced chronic inflammation and anti-inflammatory activity study were evaluated by slightly modifying the method described by (Swingle and Shideman, 1972).

Male Wistar rats, weighing 200-230 g, were divided into 5 groups of 8 rats each.

Group 1 : Control (cosolvent 5 ml/kg, p.o.).

Group 2 : Aspirin (100 mg/kg, p.o.)

Group 3 : MEKG 50 (mg/kg, p.o)

Group 4 : MEKG 100 (mg/kg, p.o)

Group 5 : MEKG 200 (mg/kg, p.o)

Subcutaneous implantation of pellets of compressed cotton provokes foreign body granuloma. Cotton rolls were cut and made into pellets weighing 20 ±1 mg each and sterilized in an hot air oven at 160°C for 2 h. Four individual pellets were inserted in each pentobarbital (35-40 mg/kg) anesthetized rats by making small subcutaneous incisions in both sides of groin regions. The incisions were sutured by sterile catgut and all process was done under sterile condition. After recovery from anesthesia, the animals were treated with cosolvent, aspirin and MEKG, orally for 7 days. On the eighth day, animals were sacrificed and the cotton pellet granulomas were freed from extraneous tissue and their wet weights were determined and then dried the cotton pellet by hot air oven at 60 °C for 18 h and weighed to obtain constant weight.

The average weight of the pellets of the control group as well as of the test group was calculated. The percent change of granuloma weight relative to control group was determined.

Inhibition of granuloma was evaluated for anti-inflammation which was expressed as % inhibition of granuloma. The percentage of inhibition was determined for each experimental group as following formula.

$$\text{Granuloma inhibition (\%)} = \frac{(W_t\text{-}W_o)_{control} - (W_t\text{-}W_o)_{treated}}{(W_t\text{-}W_o)_{control}}$$

W_t = Wet or dried cotton pellet weight

W_o = Initial of cotton pellet weight

Statistical analysis

The data obtained were analysed using SPSS software program version 11.5 and expressed as mean ± S.E.M. Statistical significance between groups was performed by the application of analysis of variance (ANOVA) followed by Bonferroni's test and Independent *t*-test for compared with 2 groups. *P* values less than 0.05 ($P < 0.05$) were used as the significant level.