

CHAPTER 3

MATERIALS AND METHODS

Extraction of medicinal plants

Piper longum dry fruit and the gall of *Quercus infectoria* were purchased from the medicinal plants store in Hat Yai, *Piper sarmentosum* root was collected in the area of Prince of Songkla University, Hat Yai Campus. The plants were cleaned with distilled water and allowed to dry under sunlight or at 50°C in a hot air oven. Then, they were pulverized and macerated individually in absolute methanol at the ratio of 1 kg of plants per 3 liters of methanol. The supernatants were collected after 7 days and the remaining plant was macerated again. This procedure was repeated twice. Whole methanol extract from each plant was filtered and evaporated under low atmospheric pressure with rotary evaporator, at 55°C.

Experimental animals

All of the animals used in this study were supplied from Division of Animal House, Faculty of Science, Prince of Songkla University, Hat Yai Campus. Female Swiss albino mice, weighing between 25-35 g, age 1-1.5 month were used for testing the effect of crude extract on caecal amoebiasis. White Wistar rat of either sex, weight between 250-300 g, and guinea-pig of either sex, weight between 400-600 g, were used for testing the effect of the crude extracts on the motility of ileum. Otherwise stated, rat pellets and water were provided *ad libitum* throughout the experiment.

Part 1. Effects of medicinal plants on amoebiasis in mice

Culture of *Entamoeba histolytica*

Entamoeba histolytica was isolated from faeces of a patient suffering from acute amoebic dysentery at Maharaj Hospital, Nakornsrihammarat

province, Thailand. The organisms were maintained in diphasic medium as described by Dutta and Rao (1966) with some modification as described elsewhere (Sawangjaroen *et al.*, 1993). Briefly, it is comprised of whole egg slants overlay with phosphate buffered saline (PBS) pH 7.4 containing 10% calf bovine donor serum and antibiotics, Penicillin G sodium (200 IU/ml) and Streptomycin sulfate (200 µg/ml). A pinch of rice starch (sterilized at 160°C for 1 h.) was added just before used. The culture tubes were incubated at 37°C. The amoebae with their associated bacteria was routinely subcultured every 24 h. Details of culture media preparation are shown in Appendix I.

Caecal amoebiasis induction in mice

Mice were starved for 24 h prior to infection with *E. histolytica*. During this fasting period, each mouse was given 0.5 ml of 25% magnesium sulfate solution orally twice, in the morning and evening (Ray and Chatterjee, 1981). On the next day, mice were anesthetized with Pentobarbital Sodium 40 mg/kg intraperitoneally (IP) and maintained with ether. Laparotomy was performed to expose the caecum. The suspension of actively motile *E. histolytica* at the volume of 0.2-0.3 ml containing 2.0×10^4 - 2.5×10^4 trophozoites was injected directly into the caecum. The caecum was then returned into the peritoneal cavity, abdominal muscle closed and skin sutured. Rat pellet were given as usual. Each mouse was randomly selected for the treatment and control group.

Preparation of crude extracts

Extract of *Quercus infectoria* was dissolved in distilled water to give an appropriate concentration. The suspension of the extract of *Piper longum* and *Piper sarmentosum* were prepared by suspending the extract in 20% gum acacia solution in distilled water. The concentration of either the aqueous solution of *Q. infectoria* or the suspension of *P. longum* and *P. sarmentosum* are 1,000, 500, 250 and 125 mg/ml. Standard drug, metronidazole tablet was

homogenized in a glass homogenizer and suspended in 20% gum acacia solution in distilled water to give the concentration of 125 and 62.5 mg/ml. Each mice was received the test agents at the volume of 10 ml/kg.

Effect of crude extracts on amoebiasis in mice

The antiamoebic effect of test materials was determined as described by Bhopale *et al.*, (1995). The methanol extract of medicinal plants were administered per os using feeding tube daily for 5 consecutive days, commencing at 24 h. after infection. The dose of 1,000, 500, 250 and 125 mg/kg body weight were used. Metronidazole, at the dose of 125 and 62.5 mg/kg body weight was used as the standard antiamoebic drug for comparison. The control animals were sham-treated with 20% gum acacia solution in distilled water (for *P. longum*, *P. sarmentosum* and metronidazole) and with distilled water (for *Q. infectoria*). Fifteen animals were used for each treatment. The animals were sacrificed by cervical dislocation at 24 h after the last dose. The abdomen was opened to remove the caecum. The morphology of the caecal walls and the presence of *E. histolytica* trophozoites were examined and scored in accordance to the criteria of Neal (1951) (See Chapter 4). In the absence *E. histolytica* trophozoites, the small amount of caecum contents were cultured in a fresh medium for 24 h and examined for trophozoites under light microscope.

Drugs and chemicals

Chemicals use in this part of experiments were penicillin G sodium and streptomycin sulfate for injection from GDH (Thailand), Metronidazole tablet was obtained from Siam Company (Thailand), gum acacia from Fluka Chemical (Neu-Ulm, Switzerland), rice starch from Sigma Chemical Company (St. Louis, USA), calf bovine donor serum from Starrate (Australia) and methanol was obtained from JT Baker Inc. (Phillipsburg, NJ, U.S.A.).

Part 2. Studies on the effects of *P. longum*, *P. sarmentosum* and *Q. infectoria* extracts on the contractility of rat or guinea-pig isolated ileum

Experimental protocols

1. Preparation of isolated rat or guinea-pig ileum

The preparation was based on the method of The Staff of the Department of Pharmacology, University of Edinburgh (1970). Rats (250-300 g) and guinea-pigs (400-600 g) of both sexes were sacrificed by cervical dislocation and exsanguination. The abdomen was opened and the caecum was exposed. The ileum was removed and cut into segments (2 cm in length). They were then transferred to a petri dish containing 95% O₂ and 5% CO₂ aerated Krebs solution. If food was present in the ileum, it was expelled by gently passing of 37°C Krebs solution through the lumen by mean of a pasture pipette. The ileal segment was then set up in an organ bath filled with 25 ml Krebs solution and aerated with 95% O₂ and 5% CO₂. Each ileum was loaded with 1 g tension and allowed to equilibrate for 30 minutes before commencement of the experiment. During the equilibration period, the ileum was washed with fresh Krebs solution every 10 minutes. The ileum contraction was recorded isometrically with a force FT03 displacement transducer connected to a Grass Model 7H polygraph (Grass International Co., Quincy, Mass. U.S.A.). Diagrammatic representation of isolated ileum preparation set up in organ bath is shown in Figure 16. Each experiment was repeated in at least 5 isolated ileums.

2. Studies on the effects of the plant extracts, loperamide, verapamil and the receptor antagonists on the contractions of rat and guinea pig ileum-induced by various spasmogens

After 30 minutes equilibration period, rat ileum was induced to contract by submaximal concentration of either acetylcholine (ACh, 10⁻⁵ M),

or serotonin (5-HT, 3×10^{-6} M). Guinea-pig ileum was used instead of rat ileum to contract by histamine (10^{-6} M) as the latter did not respond to this spasmogen. Each spasmogen was added into the bathing solution in a single concentration manner and allowed to contact to the tissues until the maximum response was achieved. The drug was then washed out and the tissue was rinsed with fresh Krebs solution. After the ileum returned to its normal state, this step was repeated until the stable response to the spasmogen was obtained. After washing out the spasmogen, the effects of the following test agents: the plant extracts, loperamide or the corresponding receptor antagonists (using atropine, chlorpheniramine and cyproheptadine to antagonize the effect of ACh, histamine and 5-HT, respectively) on the spasmogen-induced ileal contractions were then determined by addition into the bathing solution 15 minutes prior to the addition of spasmogen. These were repeated 2-4 times with higher concentrations of the test agents. In the parallel control experiment, the effects of each spasmogen (without the addition of the plant extracts, loperamide, verapamil or the receptor antagonists) was also performed in order to examine whether the responses to the spasmogen were reproducible for the period of the experiment. One ileum segment was used to test for all concentrations of one test agent.

3. Studies on the effects of the plant extracts and loperamide on the contraction of rat ileum induced by KCl 30 mM

The ileum was induced to contract by KCl 30 mM. After the stable responses to KCl were reached, the plant extracts or loperamide was then added in the bathing solution. Fifteen minutes later, KCl 30 mM was added again. This step was then repeated 2-3 times using higher concentration of the test agent. One ileum segment was used to test for all concentrations of one test agent.

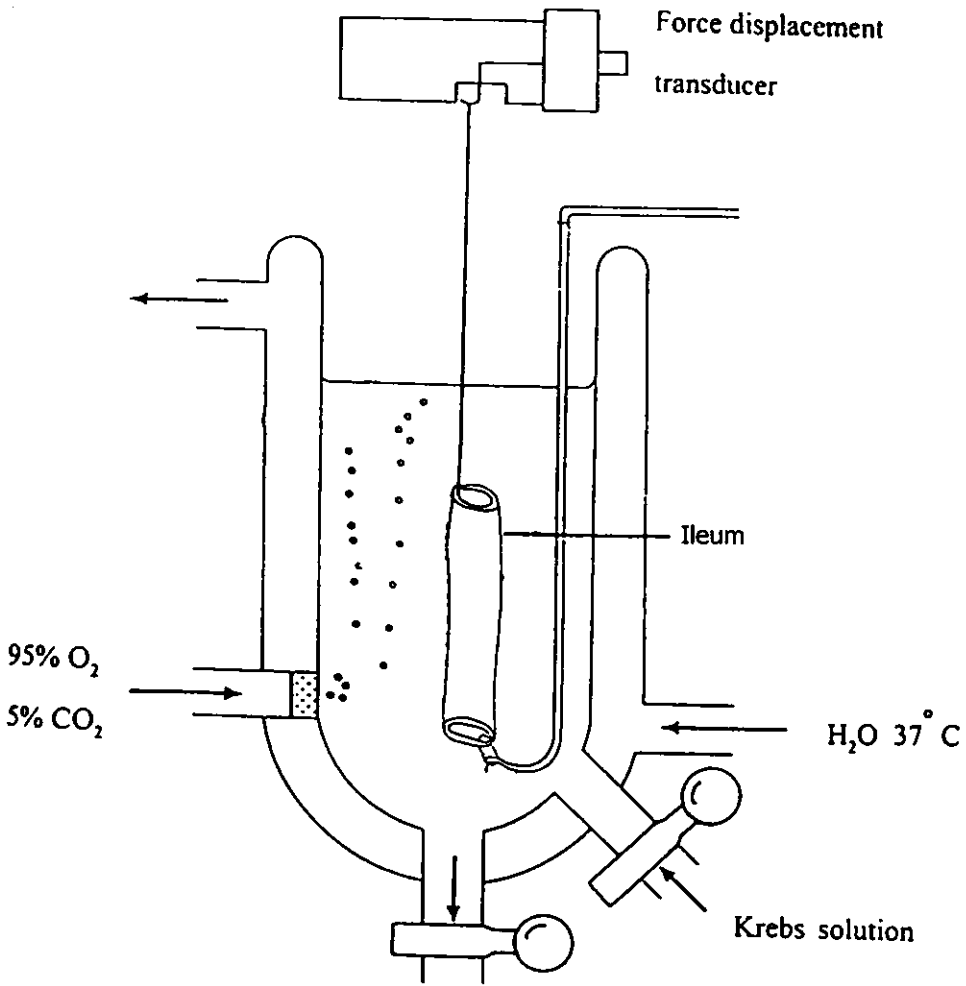


Figure 16 Set up of isolated ileum preparation in organ bath for recording of its contraction

4. Studies on the effects of the plant extracts, loperamide and verapamil on the contraction of the guinea-pig ileum induced by CaCl_2

Using the method as described by Reynolds *et al.* (1984), the isolated segment of guinea-pig ileum was set up in an organ bath containing calcium free saline solution with high potassium (KCl 80 mM). The tissue was allowed to equilibrate for 45 minutes, during which it was washed every 10 minutes with the fresh saline solution. The cumulative concentration-response relationship of CaCl_2 was then obtained by addition into the bathing solution at 2.5 minute intervals. The effect of the plant extract, loperamide or verapamil on the responses to CaCl_2 was studied as follows: each agent was added to the bathing solution immediately after washing the tissue with fresh saline solution and 20 minutes later, a cumulative concentration-response curve was then obtained. In control experiments, pairs of concentration-response curves were obtained (without the addition of the test agents) by using the vehicles to determine whether that the response was stable for the duration of the experiment. One concentration of drug was used per tissue.

Drugs and chemicals

Drugs used in this part of experiment were acetylcholine perchlorate, atropine sulphate, cyproheptadine hydrochloride, histamine diphosphate, 5-hydroxytryptamine (serotonin), loperamide hydrochloride and verapamil hydrochloride. They were purchased from Sigma Chemical Company (St. Louis, USA). Chlorpheniramine maleate (injection form) was purchased from A.N.B. Laboratory Co., LTD (Thailand). All drugs were prepared as stock solutions in distilled water and were kept at -20°C until use. On each day of experiment, working solutions were freshly diluted from the stock solution with distilled water, or DMSO to appropriate concentrations. The Krebs-Henseleit (Krebs) solution had the following composition (mM):

NaCl, 118.4; KCl, 4.7; CaCl₂, 2.9; NaHCO₃, 25; MgSO₄·7 H₂O, 1.2; KH₂PO₄, 1.2; D-glucose, 11.7; and ascorbic acid, 0.14. The high potassium and calcium free saline solution had the following composition (mM): NaCl, 55.2; KCl, 80; MgCl₂, 0.5; NaH₂PO₄, 0.4; NaHCO₃, 12 and D-glucose, 5.6. All chemicals were analytical grades.

The stock solution (1g/ml) of *Q. infectoria* methanolic extract was prepared by dissolving in distilled water while (0.1 g/ml) *P. longum* or *P. sarmentosum* was prepared by dissolving in 50% dimethyl sulfoxide (DMSO). They were kept at 4°C until use. On each day of experiment, working solutions were freshly diluted from the stock solution with distilled water (for *Q. infectoria*) or 10% DMSO (for *P. longum* and *P. sarmentosum*) to appropriate concentrations.

Statistical analysis

The results were expressed as mean \pm standard error mean. For each group, the log concentration-response curves were plotted. Regression lines were fitted to the linear portion of the log concentration response curves by method of least squares. IC₅₀ values (concentration required to produce 50% of the maximum effect) were calculated. Regression lines were tested for deviation from parallelism and relative potencies were determined.

Data were analyzed using analysis of variance (ANOVA). The analysis was performed on the individual IC₅₀ values obtained from each concentration-response curve for the test agents. When a significant heterogeneity in their estimate due to treatment was observed, further comparison of individual IC₅₀ value was made by Newman-Keuls test. Statistical significance was achieved when $p < 0.05$.