MATERIALS AND METHODS

Animals

Guinea-pigs of either sex, weighing 300 to 500 g and Sprague-Dawley rats of either sex, weighing 200 to 250 g, were supplied by the Animal Unit of the Faculty of Medicine, Chiang Mai University. Sprague-Dawley rats of either sex weighing 180 to 200 g were obtained from the National Laboratory Animal Center (NLAC), Salaya, Mahidol University, Nakornpathom. The animals were fed standard laboratory diet and water *ad libitum* and kept in an air-conditioned room at 24 $\pm$ 1° C with about 45% relative humidity. They were exposed to 12 h light and 12 h dark cycle.

Plant Material

The whole plant of *Clerodendrum petasites* S. Moore was collected in Chiang Mai, Thailand. It was identified and found to be identicaled wih a voucher specimen (QBG 4469) was deposited at the Herbarium of the Queen Sirikit Botanical Garden, Chiang Mai, Thailand.

Preparation of the ethanol extract

The whole plant of *Clerodendrum petasites* S. Moore was air-dried and milled to a fine powder. Two kilograms of the powder were macerated in ethanol (95%) and allowed to stand for 24 h. The mixture was filtered through the filter paper using a vacuum pump. The maceration was repeated 2 times. The filtrates were pooled and evaporated under reduced

pressure and controlled temperature (50 to 60°C) by using a vacuum Rotary evaporator (EYELA Tokyo Rikakikai CO, LTD.) and yielded a gummy semi-solid residue (65.7 g) which is referred to as the ethanol extract. The yield of this extract was approximately 3.29% (w/w). The test solution of the ethanol extract was prepared by dissolving the residue in 10% ethanol.

Experimental Protocols

Experiments *in vitro*

1. Isolated guinea-pig tracheal strip preparation

The method used was that described by Wellens (1966) and Lulich and Paterson (1980) with slight modification as follows:

Guinea-pigs of either sex (300 to 500 g) were killed by a blow on the back of the neck. The trachea was excised and quickly removed and placed in Krebs'solution of the following composition (g/1): NaCl 5.54, KCl 0.35, $MgSO_4.7H_2O$ 0.29, $CaCl_2$ 20.28, KH_2PO_4 0.16, $NaHCO_3$ 2.1 and glucose 2.1; cleaned off the adhering adipose and connective tissues. The tracheal strip (2 to 3 segments for each preparation) was cut open at the cartilagenous side learing the smooth muscle section in the middle of the resulting tracheal strip. The strip was then mounted in a 20 ml organ bath containing the Krebs' solution continuously aerated with a mixture of 95% O_2 and 5% CO_2 and maintained at 37° C. The tracheal strip preparation was allowed to equilibrate under 1 g resting tension for 60 to 90 min, during this time the bath solution was replaced every 15 min. Isometric tension

change was recorded via a force–displacement transducer (Grass FT 036, Grass Instrument Co. Quincy, Mass., U.S.A.) and displayed on a polygraph (79D Polygraph Grass Instrument CO. Quincy, Mass., U.S.A.) as shown in Figure 2. After finishing each dose of test drug, the tissue was washed 3 to 4 times with fresh Krebs' solution and allowed to return passively to its resting tension before being reused.

In this study, histamine and acetylcholine were used as a bronchoconstrictive inducer. The dose of histamine and acetylcholine which caused the submaximal contraction of the tracheal muscle was first determined and used to induce bronchoconstriction.

2. Quantitive measurement of tracheal activity

The tracheal contraction induced by a submaximal effective concentration of histamine or acetylcholine represents bronchoconstriction.

The bronchodilator effect of the ethanol extract and reference drugs (terbutaline, atropine, aminophylline and papaverine) was tested when the agonist-induced tracheal contraction reached a plateau which usually occurred within 5 to 7 min. The antagonistic effect of the ethanol extract and reference drugs was expressed in term of the percent relaxation.

$$\% \text{ relaxation} = \frac{R_1 - R_2}{R_1} \times 100$$

where R_1 = response to histamine or acetylcholine

R_2 = response after adding test drug

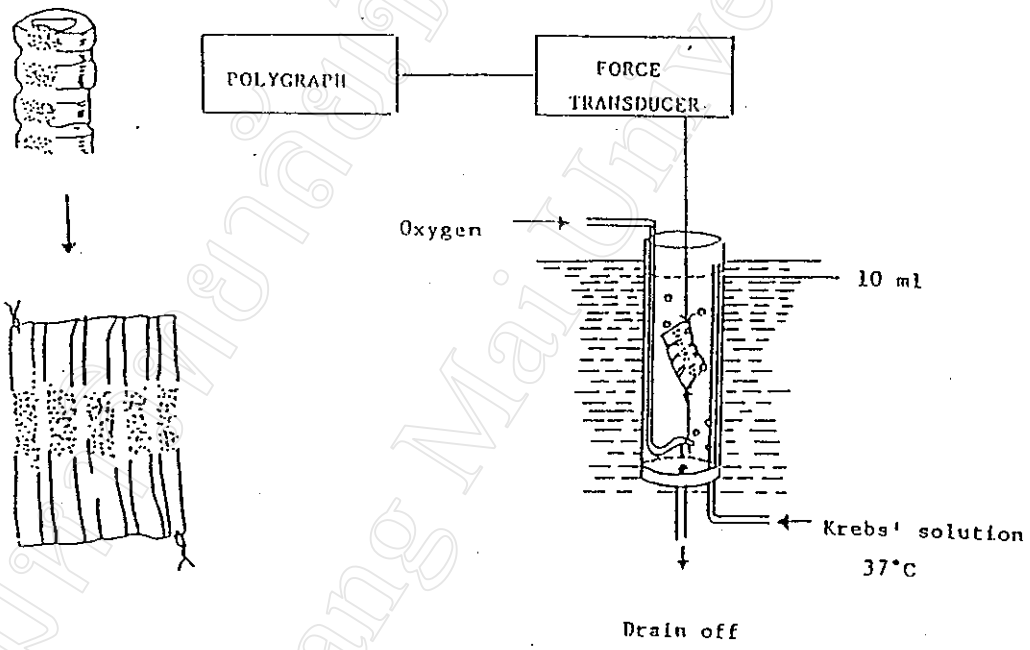

Figure 2. Experimental set for recording of tracheal contraction using isolated guinea-pig tracheal strip

The potency of the ethanol extract was assessed by comparison of its median effective concentration (EC_{50}) value with those of reference drugs. The value of EC_{50} was defined as the concentration of test drug which induced a 50% relaxation of the agonist-induced tracheal contraction.

3. Determination of the mechanism of bronchodilator action of the ethanol extract from C. petasites

The possible mechanisms of action of the ethanol extract were investigated by:

3.1 Comparison of the dose-response relationship of the ethanol extract with reference drugs on the histamine- and acetylcholine-induced tracheal contraction

By employing a least square method, the dose-response relationship between various doses of the ethanol extract or reference drugs and their percent relaxation of the histamine or acetylcholine-induced tracheal contraction was expressed as a linear regression equation, $Y = a + bX$; and their slopes were tested for parallelism.

3.2 Comparison of the antagonist effect of the ethanol extract and reference drugs on the histamine-induced tracheal contraction in the presence of propranolol (β-adrenergic antagonist)

Doses of reference drugs and the ethanol extract which caused nearly the same percent reduction of histamine-induced tracheal contraction (95 to 100%) were used. The dose of propranolol which could effectively

block the effect of terbutaline (β_2- adrenergic agonist) was determined and used to study its blocking effect on the reduction of a histamine-induced tracheal contraction caused by aminophylline, papaverine and ethanol extract. In this study, histamine was added into the chamber 3 min after the tracheal muscle was pretreated with propranolol. The antagonistic effect of the test drugs was then evaluated when the histamine-induced tracheal contraction reached a plateau. The reduction of the histamine-induced tracheal contraction occurring in response to the test drugs when propranolol was either absent or present, was recorded.

3.3 Determination of the blocking effect of the ethanol extract on acetylcholine-induced bronchoconstriction in compairison with atropine (antimuscarinic agent).

A dose of the ethanol extract and of atropine which caused maximum relaxation of acetylcholine-induced contraction was used. The blocking effect of the ethanol extract and atropine was assessed by adding each of them into the organ bath 3 min before challenge with acetylcholine. If the tracheal muscle produced no response to acetylcholine, histamine was then added onto the organ bath 3 min thereafter.

Experiments *in vivo*

1. Histamine-induced bronchoconstriction in pentobarbital anesthetized guinea-pig

The experiment was performed according to the method described by Salonen (1985) and slightly modified as follows:

Guinea-pigs of either sex (300 to 500 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg). Polyethylene tube was inserted into the trachea and the right jugular vein was cannulated for the administration of drugs. The animal was artificially ventilated with a Harvard rodent ventilator (Model 680, Harvard Apparatus Co., Dover, Mass., U.S.A.) and adjusted to a tidal volume of 7 ml/kg at the ventilation rate of 70 strokes/min. Intratracheal pressure was measured by connection of a side arm of the tracheal cannula to a bronchospasm transducer (720 model, Ugo Prasile, Italy), the output of which was fed to preamplifier of a polygraph (79D Polygraph Grass Instrument Co., Quincy, Mass., U.S.A.) as shown in Figure 3. The amplitude of the penwriter was adjusted to give 2 ml of air equal to 1 cm. An increase in the intratracheal pressure was taken to denote bronchoconstriction. The blood pressure of the animal was concurrently recorded from the left common carotid artery using a pressure transducer (Statham P23 AC Strain gauge Transducer, Laboratory Inc., Hato Rey, Puerto Rico) connected to the polygraph. To prevent spontaneous breathing, 2 mg/kg of pancuronium bromide was given intraperitoneally. During the experiment no extra dose of anesthetic or relaxant was given. The animals failed to show full anesthesia with

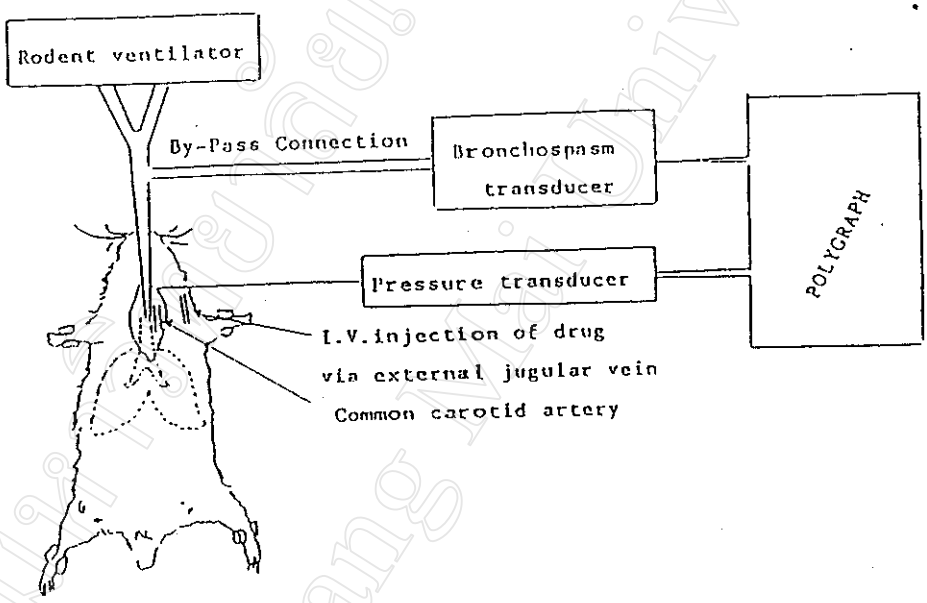

Figure 3. Experimental set for recording of intratracheal pressure in pentobarbital anesthetized guinea-pigs or rats

complete muscle relaxation were discarded. About 10 min after the administration of muscle relaxant, the animal usually showed no signs of spontaneous breathing.

In each guinea-pig, two supramaximal doses of histamine were used to induce bronchoconstriction (Drazen and Austen, 1974). Only animals which responded to the first dose of histamine were used. The second dose of histamine was injected 20 min after the first dose. The ethanol extract and reference drugs as well as control vehicle were given 2 min before the second dose of histamine. After each dose of the drugs the polyethylene catheter was rinsed with 0.1 ml of normal saline solution (NSS).

2. *Quantitative measurement of bronchial activity*

Quatitative measurement of bronchoconstriction was assessed from the difference in the penwriter amplitude between peak intratracheal pressure response (PIPR) induced by histamine and the basal resting value. A single animal was used for a single dose of the test drugs. The bronchodilator activity of the test drugs was expressed as a percent inhibition of PIPR to the second dose of histamine-induced bronchoconstriction which was calculated as follows:

$$\text{Bronchodilator activity} = \frac{B_1 - B_2}{B_1} \times 100$$

where B_1 = bronchoconstriction induced by histamine

B_2 = bronchoconstriction induced by histamine after pretreatment with test drug

3. Methacholine-induced bronchoconstriction in pentobarbital anesthetized rat

The experiment was performed following the method described by Salonen and Mattila (1981) and slightly modified as follows:

Sprague-Dawley rats of either sex (200 to 250 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg). Polyethylene tube was inserted into the trachea and the right jugular vein was cannulated for the administration of drugs. The animal was artificially ventilated with a Harvard rodent ventilator (Model 680, Harvard Apparatus Co., Dover, Mass., U.S.A.) and adjusted to a tidal volume of 10 ml/kg at the ventilation rate of 70 strokes/min. Intratracheal pressure and blood pressure were measured using the method similar to that performed in guinea-pigs.

A set of doses of methacholine (MeCh) was injected (1.5, 3 and 4.5 μg) at 2 min interval to induce bronchoconstriction. Only animals which responded to the first set of cumulative doses of MeCh were used. The second set of MeCh doses (3, 6 and 9 μg) was injected 20 min after the first set of MeCh dose, also at 2 min interval. The ethanol extract and reference drugs as well as control vehicle were given 2 min before the second set of cumulative doses of methacholine. After each dose of the drug, the polyethylene catheter was rinsed with 0.1 ml of NSS.

The antagonistic effect of the ethanol extract and reference drugs on MeCh-induced bronchoconstriction was determined by comparing the PIPR in the response to the second set of MeCh-induced between drug-treated group and control group (NSS).

4. Hippocratic screening test

Documentation of observed symptoms after various doses of ethanol extract was recorded and graded according to the method described by Malone and Robichaud (1962).

Non-fasted Sprague-Dawley rats of either sex (180 to 200 g) were used. Four animals were used for each dosage level of the ethanol extract. Signs and symptoms induced by an intraperitoneal injection of the ethanol extract were observed at 5, 15, 30, and 60 min; 2, 4 and 6 h after administration. The changes in various behaviors were recorded on the standard work sheet (Figure 4). Five dosage levels of the ethanol extract were used, the lowest dose was the ineffective dose and the highest dose was the lethal dose. The other three doses were between the lowest and the highest doses. After 7 days of observation, all alive animals were sacrificed and necropsy was performed to examine the internal organs (heart, lungs, livers, kidneys, intestine, etc.) for any unusual signs (e.g. change in colour and size) in comparison with those of control animals which received a control vehicle. An animal which died acutely from drug effect, was autopsied to examine the internal organs.

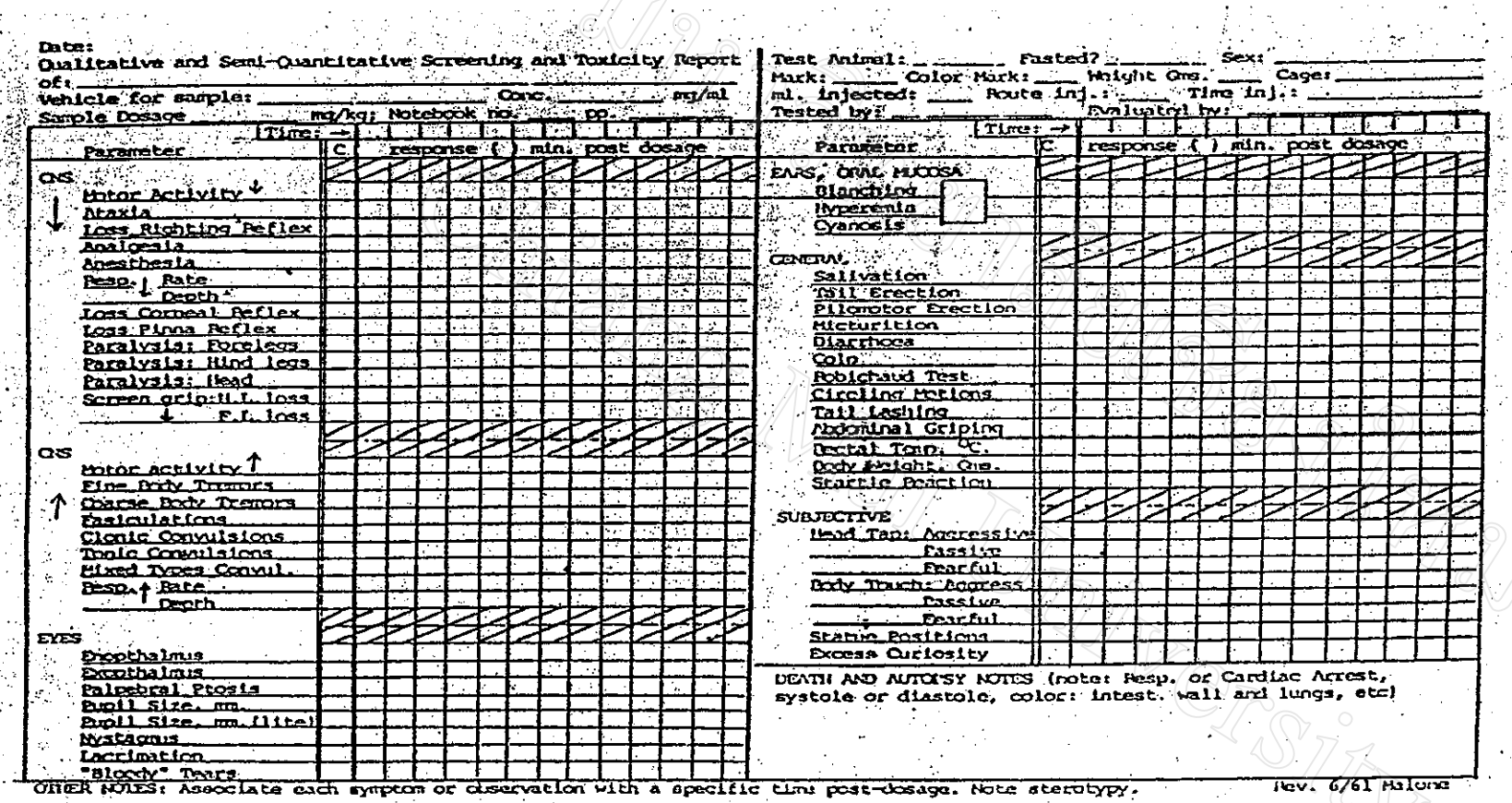

Figure 4. Standard work sheet for Hipporcratic screening test

Statistical analysis

The data are expressed as mean $\pm$ S.E.M. The following statistical methods were used in evaluation of the data

1. Student's t-test, for assessing the significance of differences between the two group, $p < 0.05$ is considered significant
2. Correlation coefficient value (r), for evaluating the association of dosage and response
3. Linear regression equation (Y = a + bX), for evaluation median effective concentration (EC_{50}) or median effective dose (ED_{50}), the concentration or dose which produced 50% response

Drugs and chemicals

1. Acetylcholine (Calbiochem, Los Angeles, U.S.A.)
2. Aminophylline, 250 mg/10 ml ampule (Atlantic laboratory, Thailand)
3. Atropine sulfate U.S.P. XIX, 0.60 mg/ml ampule (The Government Pharmaceutical Organization, Thailand)
4. Chlorpheniramine maleate, 10 mg/ml ampule (Piriton^{R}, Schering, Germany)
5. Heparin 5000 i.u./ml vial (Leo Pharmaceutical Product, Ballerup, Denmark)

6. Histamine dihydrochloride, B grade (Calbiochem, Los Angelis, U.S.A.)
7. Methachloline choride (Acetyl-B-methacholine chloride) (Sigma Chemical Company, St. Louis, U.S.A.)
8. Pancuronium bromine, 2 mg/ml ampule (Pavulon^{R} sodium solution, Organon Oss, Holland)
9. Pentobarbital sodium, 50 mg/ml vial (Nembutal^{R} sodium solution, Abbott Laboratories, North Chicago, U.S.A.)
10. Papaverine hydrochloride powder (Ingelheim am Rhein, Germany)
11. Propranolol hydrochloride, 1 mg/ml ampule (Inderal^{R}, I.C.I. Macclefield, Great Britain)
12. Terbutaline sulphate, 0.5 mg/ml ampule (Bricanyl^{R}, Astra, Sweden)
13. Absolute ethanol (J.T. Baker Chemical B.V., Holland)