

CHAPTER 3

MATERIALS AND METHODS

1. Plant Material Preparation

The fresh leaves of *Carica papaya* L. (20 kg) were obtained from a papaya plantation, Ronpiboon District, Nakhonsithammarat Province, Thailand, in January 2001. The plant material was identified by a botanist of Botany Section, Department of Biology, Prince of Songkla University. They were cleaned with distilled water. Only green leaves which do not have any scars or spot of disease were selected for further extraction. Some large veins of the leaves were removed. The leaves were then air-dried at room temperature overnight, and were further dried in hot air oven at 50 °C until dryness. The dried leaves were pulverized to coarse powder using an electric blender. The powder (2.2 kg.) was then used for alkaloid extraction according to the solvent extraction technique by Cordell, (1981).

2. Drugs and Chemicals

Acetylcholine chloride, (±)-verapamil hydrochloride, DL-propranolol hydrochloride, (±)-isoproterenol hydrochloride, dimethylsulfoxide (DMSO) and diethylstilbestrol (DES) were purchased from Sigma Chemical Company (St. Louis, U.S.A.). All drugs, except DES were prepared as stock solutions (acetylcholine 10^{-1} M, verapamil 10^{-4} M, propranolol 10^{-4} M, and isoproterenol 10^{-1} M) in 0.1% ascorbic acid in distilled water. DES was dissolved in olive oil to give a concentration of 1 mg/ml for intraperitoneal injection. Oxytocin

(syntocinon) was purchased from Novartis and Pharmacia and $\text{PGF}_{2\alpha}$ (Dinoprost thromethamine) from Upjohn (Belgium). Papaya leave's alkaloid (PA) was dissolved in dimethylsulphoxide (DMSO) to give a stock solution (200 mg./ml) for use in all experiments. The final concentration of DMSO in the organ bath was less than 0.5% and did not affect contraction or relaxation. NaCl and NaHCO_3 were purchased from Merck, KCl, CaCl_2 and glucose were purchased from Carlo Erba. These common salts were used to prepare physiological solution, (see Appendix I), which was freshly prepared for each day of experiment.

3. Extraction Procedure

The extraction procedures of the crude alkaloid from *Carica papaya* L. leaves are as follows. 1) The papaya leave powder was moistened with concentrate NH_4OH solution. 2) It was then macerated in 20 liters of absolute methanol for 3 days at room temperature, the methanol extract was then removed, filtered and stored at 4 °C. 3) The extraction was repeated for 7 times or until no trace of alkaloid was detected using Dragendroff's reagent. 4) The whole methanolic extracts were pooled, and evaporated at 50 °C under reduced pressure in Rotavac Evaporator (Buchi®). 5) The extract (1,300 ml black viscous, oil-like mixture) was dissolved in 1,300 ml of 2% sulfuric acid in water and then extracted with 2,600 ml of hexane to removed fat materials. 6) The acid fraction was adjusted to pH 9 using concentrate NH_4OH solution and further extracted with excess chloroform. 7) The chloroform fractions were then pooled and evaporated at 50 °C under reduced pressure until dryness. 8) The crude alkaloid extract obtained from step 7 was dissolved in

2% sulfuric acid in water and the extraction procedure from step 6 and step 7 were repeated. The crude alkaloid extracts (9.1 g) were obtained and stored in an air tight bottle at 4° C until use. Diagrammatic representation of the alkaloid extraction procedure is shown in Figure 18.

4. Experimental Animals

All experiments were carried out using virgin female Wistar rats (200-300 gm.) which were supplied from Animal House, Faculty of Science, Prince of Songkla University. They were housed in air-conditioned room (24-26 °C) with a 12 hours light/dark cycle. Rats were injected intraperitoneally with diethylstilbestrol (DES) dissolved in olive oil (100 µg/ml) 24 hours before the experiment.

5. Experimental Protocol

The rats were euthanased by cervical dislocation, uterine horns^x removed and placed in a Jalon-Ringer solution. Each horn was cut into a segment of approximately 1 cm. in length. The uterus segment was then mounted in 20 ml organ-bath chamber filled with Jalon-Ringer solution. This medium was gassed continuously with 95% O₂, 5% CO₂ and maintained at 37 °C throughout the experiments. The uterus was set up under initial resting tension of 2 g. Changes in uterine tension were recorded isometrically with a force displacement transducer (Grass Instrument CO, Quincy, Mass, U.S.A.) connected to a Grass Model 79 D polygraph. Before the commencement of each experiment, the uterus preparation was equilibrated for at least 1 hour and the Jalon-Ringer solution in the organ bath was changed periodically with fresh Jalon-Ringer solution. In all experiments, a uterus segment from the

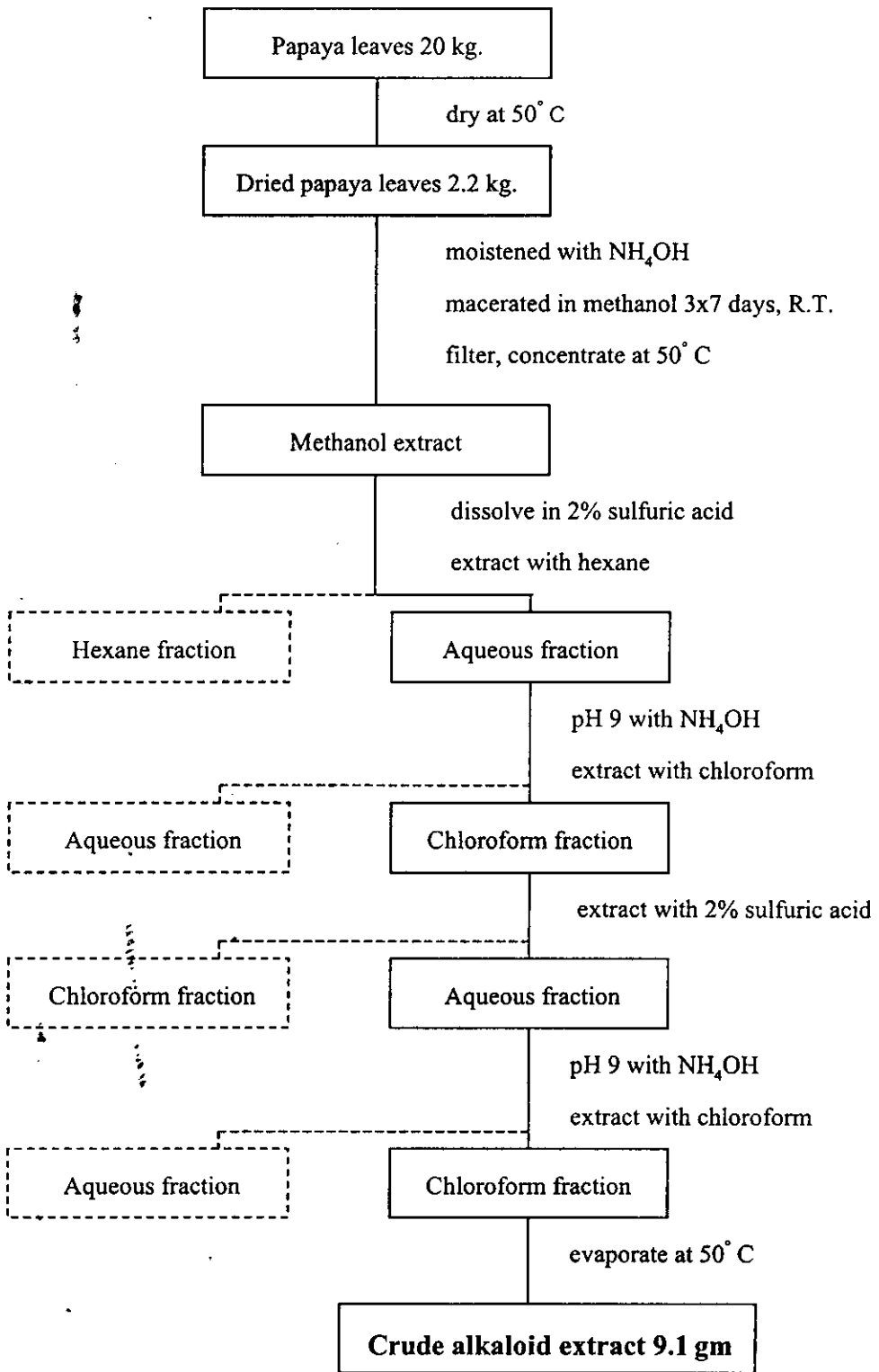


Figure 18. The extraction process for crude alkaloid from *Carica papaya* L. leaves

other horn was also set up as a time control preparation to compare the effect of time to the drug effect and/or vehicle. This uterus was prepared and used in the same manner as the drug treated uterus, but the relevant vehicle of the drug was added to the organ bath instead of the drug solution. Each experiment was repeated in at least 7-10 replicates.

6. Experimental Procedures

6.1 Effect of PA on uterine contraction

The uterine horn was equilibrated for 1 hours in Jalon-Ringer solution under resting tension of 2 gm. and maintained at 37 °C. After then, PA (10^{-6} - 10^{-3} gm/ml) were added cumulatively to the organ bath. Changes in uterine tension were recorded as described in experimental protocol.

6.2 The determination of optimal KCl concentration in depolarizing solution

The uterus was immersed in Jalon-Ringer solution and equilibrated for 1 hour under resting tension of 2 gm. After the equilibration period, the uterus was induced to contract by changing the solution in the bath to either of depolarizing solutions containing 36.3, 46.3, 56.3, 66.3 or 76.3 mM of KCl. The effect of each depolarizing solution was allowed to reach maximum and the contraction was observed for a further of 10-minute period. The uterus was then washed three times with fresh Jalon-Ringer solution before the other depolarizing solution was replaced. Changes in uterine tension were recorded and concentration-response curves were constructed. From this experiment, a depolarizing solution containing 56.3 mM of KCl was chosen for use in the following experiments. The addition of this depolarizing solution caused a

rapid contraction, followed by a slight relaxation and prolonged contraction plateau.

6.3 Effect of PA on uterine contraction induced by depolarizing solution

This experiment was performed using K^+ -depolarized uterus. The uterus was immersed in Jalon-Ringer solution and equilibrated for 1 hour under resting tension of 2 gm. After the equilibration period, the uterus was contracted by changing the solution in the bath to a depolarizing solution (56.3 mM KCl). When the contraction plateau was reached, cumulative concentrations of PA were added to the organ bath. The relaxation was allowed to reach maximum before the addition of the next concentration of PA. The time control treatment was performed in parallel to that of the PA treatment. The total volume of drug added was kept as small as possible with a maximum allowance volume of 1 ml. Changes in uterine tension were recorded and the log concentration-response curves were then constructed.

6.4 Determination of optimal concentration of verapamil

The uterine horns were bathed for 1 hour in Jalon-Ringer solution with resting tension of 2 gm. After then, the solution was replaced by Ca^{2+} -free high K^+ solution (KCl 60 mM, see Appendix I) and the uterus was washed with this solution for at least 3 times in 10-minute intervals and then the uterus was induced to contract by adding 10^{-3} M of $CaCl_2$. When the plateau contraction was reached, cumulative concentrations of verapamil were added to the organ bath. The relaxation was allowed to reach maximum before the addition of the next concentration of verapamil. The time control treatment was performed in

parallel to that of the verapamil treatment. Changes in uterine tension were recorded and the log concentration-response curves were then constructed. The concentration of verapamil (10^{-7} M) which produced uterine relaxation to approximately 80 - 90% of maximum contraction was selected to use in the next experiment.

6.5³ Effect of PA or verapamil on uterine contraction induced by CaCl_2 in K^+ -depolarized uterus

The uterine horns were bathed for 1 hour in Jalon-Ringer solution with resting tension of 2 gm. After then, the solution was replaced by Ca^{2+} -free high K^+ solution (KCl 60 mM, see Appendix I) and the uterus was washed with this solution for at least 3 times in 10-minute intervals until the baseline tension was achieved. Cumulative concentrations of CaCl_2 (10^{-5} - 3×10^{-2} M) were then added. After the uterine contraction reached a maximum, the uterus was then washed using Ca^{2+} -free high K^+ solution for many times. After uterine tension returned to the baseline position, the uterus was then preincubated with either PA (10^{-5} gm/ml) or verapamil (10^{-9} M) followed by cumulative concentration of CaCl_2 (10^{-5} - 3×10^{-2} M). This experiments were repeated but the concentration of PA or verapamil was changed to 3×10^{-5} and 10^{-4} for PA or 10^{-8} or 10^{-7} M for verapamil respectively. The uterine tension in response to each concentration of CaCl_2 in the present or absence of PA or verapamil was recorded and the concentration response curves were constructed.

6.6 Determination of optimal concentration of oxytocin, $\text{PGF}_{2\alpha}$ and acetylcholine

The uterine horn was equilibrated for 1 hour in Lock-Ringer solution under resting tension of 2 gm. and maintained at 37°C . The uterus was induced to contract using, oxytocin (0.01-10.0 mU/ml), $\text{PGF}_{2\alpha}$ (10^{-9} - 10^{-4} M) or acetylcholine (10^{-7} - 3×10^{-4} M) in a cumulative concentration manner. The tension responses of the uterus to the stimulants were recorded and the concentration-response curve of each stimulant was then constructed. The concentration of each stimulant which produced uterine contraction approximately 70-80% (oxytocin (1 mU/ml), ACh (3×10^{-5} M), $\text{PGF}_{2\alpha}$ (10^{-5} M) of the maximum contraction was selected for use in the next experiments.

6.7 Effect of PA or verpamil on uterine contraction induced by oxytocin.

A uterine horn was incubated in Lock-Ringer solution with resting tension of 2 gm. for 1 hour at 37°C . The uterus was precontracted submaximally by the addition of oxytocin (1mU/ml). After the rhythmic contraction was stable, PA (10^{-6} - 10^{-3} gm/ml) or verapamil (10^{-8} - 10^{-4} M) were then added cumulatively to the organ bath. The uterine tension in response to each concentration of PA or verapamil was allowed to develop maximally before the addition of the next concentration. The time control treatment was performed similarly to that of PA or verapamil treatment and both treatment were performed in parallel. Change in force and frequency of contraction was recorded. The force of contraction was the value of the maximum change of

the uterine tension in response to each concentration of PA or verapamil. The frequency of rhythmic contraction was calculated by averaging the number of contraction within 15 minutes. The log concentration-response curves of force or frequency of contraction were then constructed.

6.8 Effect of PA on uterine contraction induced by $\text{PGF}_{2\alpha}$

A uterine horn was incubated in Lock-Ringer solution with resting tension of 2 gm. for 1 hour maintained at 37 °C. The uterus was precontracted by the addition of $\text{PGF}_{2\alpha}$ (10^{-5} M). After the rhythmic contraction was stable, PA (10^{-6} - 10^{-3} gm/ml) were then added cumulatively to the organ bath. The response to each concentration of PA was allowed to reach maximum before the addition of the next concentration of PA. The time control treatment was also performed similarly to that of PA treatment and both treatment was performed in parallel. Change in force and frequency of contraction was then recorded.

6.9 Time-course relationship of PA or verapamil on uterine contraction induced by acetylcholine

The uterus was equilibrated for 1 hour in Lock-Ringer solution under resting tension of 2 gm and maintained at 37 °C. After the equilibration period, acetylcholine (3×10^{-5} M) was added to the organ bath. After the uterine contraction reached a maximum, the uterus was then washed using Lock-Ringer solution for at least 3 times. The experiment was then repeated but before adding acetylcholine (3×10^{-5} M), the uterus was preincubated with PA (10^{-4} gm/ml) or verapamil (10^{-6} M) for various periods of time (5, 10, 15, 20 and 25 minutes). The time control treatment was performed similarly to that of

PA or verapamil treatment and both treatments were performed in parallel. The response of uterus to acetylcholine (3×10^{-5} M) in the presence or absence of PA (10^{-4} gm/ml) or verapamil (10^{-7} M) was recorded and the time-response relationships were then constructed. The optimal incubation time of PA or verapamil which produced maximum effect on acetylcholine-induced contraction was selected for used in the next experiments.

6.10 Effect of PA on uterine contraction induced by acetylcholine

A uterine horn was incubated in Lock-Ringer solution with resting tension of 2 gm for 1 hours maintained at 37° C. A single dose of acetylcholine (3×10^{-5} M) was then added to induce uterine contraction. After the contraction reached the maximum, the uterus was washed using fresh Lock-Ringer solution. This experiment was repeated but 15 minutes before adding acetylcholine, various concentrations of PA (10^{-6} - 10^{-3} gm/ml) or verapamil (10^{-8} - 10^{-4} M) was added. The time control treatment was performed similarly to that of PA or verapamil treatment and both of treatment was performed in parallel. Changes in uterine tension were recorded and the concentration-response curves were then constructed.

It is noted that the effect of acetylcholine on the rhythmic contraction of the uterus was unstable as can be seen by a dramatically gradual decrease in both force and frequency of contraction from time control. This would make and erratic interpretation whether the effect was due to PA or verapamil on the common effect of acetylcholine when cumulative concentration of PA or verapamil was used. To overcome this problem, the experimental design was improved by preincubating the uterus with a single concentration of either PA

or verapamil before acetylcholine was added. The force of uterine contraction is measured by a maximum of first peak of contraction.

6.11 Effect of PA or verapamil on oxytocin-induced contraction in Ca^{2+} -free solution

Uterine horn was equilibrated for 1 hour in Lock-Ringer solution under a resting tension of 2 gm maintained at 37 °C. The solution was then replaced by Ca^{2+} -free solution containing 2 mM EDTA and the incubation was continued for a further of 50 minutes. Subsequently, the solution was replaced by Ca^{2+} -free solution containing 0.02 mM EDTA (see Appendix I) and the uterus was incubated for a further of 20 minutes. Oxytocin (10 mU/ml) was then added to the organ bath. After the tonic contraction of the uterus reached the maximum, cumulative amounts of PA (10^{-6} - 10^{-3} gm/ml) or verapamil (10^{-8} - 10^{-4} M) were added. The time control treatment was performed similarly to that of PA or verapamil treatment and both of treatment was performed in parallel. The uterine tensions in response to each concentration of PA or verapamil were recorded and the concentration-response curves were then constructed.

6.12 Determination of optimal concentration of propranolol

This experiment was performed using K^{+} -depolarized uterus. The uterus was immersed in Jalon-Ringer solution and equilibrated for 1 hour under resting tension of 2 gm. After equilibration period, the uterine contraction was induced by changing the solution in the bath to a depolarizing solution (KCl 56.3 mM). This addition caused a rapid contraction, followed by a slight relaxation and prolonged contraction plateau. When the plateau was reached,

cumulative concentrations of isoproterenol (10^{-10} – 10^{-5} M) were added. After the uterus has reached a maximum relaxation, the uterus was washed using Jalon-Ringer solution for many times. The experiment was then repeated but before adding isoproterenol, either a single concentration of propranolol (10^{-8} , 10^{-7} and 10^{-6} M) was added for 10 minutes. The time control treatment was performed similarly to that of propranolol treatment and both of treatment was done in parallel. The effect of each concentration of isoproterenol in the presence or absence of propranolol was recorded and the concentration-response curves were then constructed. The concentration of propranolol (10^{-7} M) which produced 70-80% inhibition of the maximum effect of isoproterenol was selected for used in the next experiments.

6.13 Time course-response relationship of propranolol on isoproterenol induced uterine relaxation

This experiment was performed to find out an optimum incubation period of propranolol. The uterus was immersed in Jalon-Ringer solution and equilibrated for 1 hour under resting tension of 2 gm. After equilibration period, the uterus was contracted by changing the solution in the bath to a depolarizing solution (KCl 56.3 mM). When the tonic contraction plateau was reached, cumulative concentrations of isoproterenol (10^{-10} – 10^{-5} M) were added to the organ bath. After the uterus has reached a maximum relaxation, the uterus was then washed using Jalon-Ringer solution for many times. The experiment was then repeated but before adding isoproterenol, the uterus was incubated with propranolol (10^{-7} M) for various periods of time (10, 20 and 30 minutes). The time control treatment was performed similarly to that of the

propranolol treatment and both treatment was done in parallel. The effect of each concentration of isoproterenol in the presence or absence of propranolol (10^{-7} M) was recorded and the concentration-response curves were constructed. The optimal incubation time of propranolol which produced maximum effect was chosen for use in the next experiments.

6.14 Effect of propranolol on uterine relaxation induced by PA or isoproterenol

The uterus was immersed in Jalon-Ringer solution and equilibrated for 1 hour under resting tension of 2 gm. After equilibration period, the uterus was induced to contract by changing the solution in the bath to a depolarizing solution (KCl 56.3 mM). When the tonic contraction plateau was reached, cumulative concentrations of isoproterenol (10^{-10} – 10^{-5} M) or PA (10^{-6} – 10^{-3} gm/ml) were added to the organ bath. After the uterus reached a maximum relaxation, the uterus was then washed using Jalon-Ringer solution for many times. The experiment was then repeated but before adding isoproterenol or PA, the uterus was preincubated with propranolol (10^{-7} M) for 20 minutes. The time control treatment was performed similarly to that of PA or isoproterenol treatment and both treatments were performed in parallel. The effect of each concentration of PA or isoproterenol in the presence or absence of propranolol (10^{-7} M) on uterine contraction induced by depolarizing solution was recorded and the concentration-response curves were then constructed.

7. Data Analysis

Contractile responses of the uterus were determined as a change in isometric tension (in gm), that induced by each stimulant (acetylcholine, oxytocin, CaCl_2 , $\text{PGF}_{2\alpha}$, and depolarizing solution) or uterine relaxant (isoproterenol, verapamil) or PA. All data were expressed as mean \pm standard error (Mean \pm S.E) of percentage of the maximum contraction induced by each stimulant. Inhibition concentration 50% (IC_{50}) was calculated graphically from a plot of log concentration vs. the maximum response (E_{max}) produced by each uterine relaxant in individual experiment. Statistical significance of differences between each group of means was assessed by repeated measure analysis of variance (ANOVA). In case that there was a significant difference among group of means, multiple comparison among means were then performed using Duncan's multiple range test. A significant difference will be determined when P is less than 0.05.