CHAPTER III

MATERIALS AND METHODS

1. Laboratory Animals

Male Wistar rats weighing 250-300 g were purchased from The National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Pathom, Thailand.

The animals used in this study were housed in animal care facility at the Faculty of Pharmaceutical Sciences, Chulalongkorn University, in a room under controlled environment (12-h light-dark cycle, lights on at 7:00 am; temperature at 25 $\pm$ 1°C) before experiment at least 1 week. Animals were provided with standard diet and water ad libitum. This study was approved by The Ethics Committee of The Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

2. Experimental instruments

1. Organ Bath (Double Walled Harvard Type, 25 ml)

2. Water Bath (model HetoOBN18) consist of Thermoregulating Water Pump (model HWT100 from Johan Nordic, Gydeuang, Denmark)

3. Isometric force transducer model MLT050/A (AD Instrument, Castle Hill, Australia)

4. PowerLab/4SP equipped connected to a computer with program SCOPE CHART 5 V. 2.0 (AD Instrument, Castle Hill, Australia)

5. Tank of carbogen gas (95% O_2, 5% CO_2) (T.I.G, Bangkok, Thailand)

6. Weighing machine (METTLER AB204-S, AG135)

7. Minor Surgery Instruments

3. Drugs and Chemicals

Passiflora foetida L. (PF) were extracted by hexane (PF-002) and by 90% methanol (PF-003). All the *P. foetida* extracts (Fraction 002: Sub-fractions PF002-(1-4), PF002-5, and PF002-7 and fraction 003: PF003-1, PF003-2 and PF003-(3-5)) were kindly provided by Associate Professor Rutt Suttisri, Ph.D., Department of Pharmaceutical Botany, Faculty of Pharmacetical Sciences, Chulalongkorn University. All extracts were kept at -20^{o}C and were dissolved in dimethy sulfoxide (DMSO, 99.5% V/V) until used. On the day of experiment, the stock solution of extracts was freshly dissolved in 99.5% DMSO before used.

Other chemicals including norepinephrine, propranolol, 5-hydroxytryptamine (serotonin), caffeine, ketanserin, tyramine and reserpine were obtained from Sigma Chemical, Missouri, USA.

4. Experimental methods

4.1 Preparation of rat isolated atria

Male Wistar rats (250-300 g) were sacrificed by cervical dislocation. The heart was rapidly removed, and the atria were dissected from the heart. Both left and right atria were excised and mounted immediately in 20 ml. organ bath which contained Krebs-Henseleit Solution of the following composition (mM): NaCl 118.0, KCl 4.7, $CaCl.2H_2O$ 2.52, $MgSO_4.7H_2O$ 1.64, $NaHCO_3$ 24.88, KH_2PO_4 1.18 and glucose 11.10.

This solution was maintained at 37 ^{o}C, pH 7.4 and continuously bubbled with 95% O_2 and 5% CO_2.

The isolated left and right atria were attached to isometric force transducers (model MLT050/A, AD Instrument, Castle Hill, Australia) with a 0.5 gram initial load of resting tension. The left atria were also connected with electrical stimulation at 4.2 Hz, impulse of 5 ms duration with threshold at 5 volts to induce contraction by electrical pacing. The transducer was coupled to powerlab/4SP, AD Instrument, Castle Hill, Australia, which was connected to the computer equipped with program SCOPE CHART 5 V. 2.0 (AD Instrument, Castle Hill, Australia) to analyze the rate and force of contraction. The right atria were allowed to beat spontaneously. The tissues were equilibrated for at least 30 min or until the tension was stable before the application of any drug (Figure 7). The basal contractile activities were referred 100%. The response were calculated as a change in percentage of contraction or heart rate from the basal contractivities.

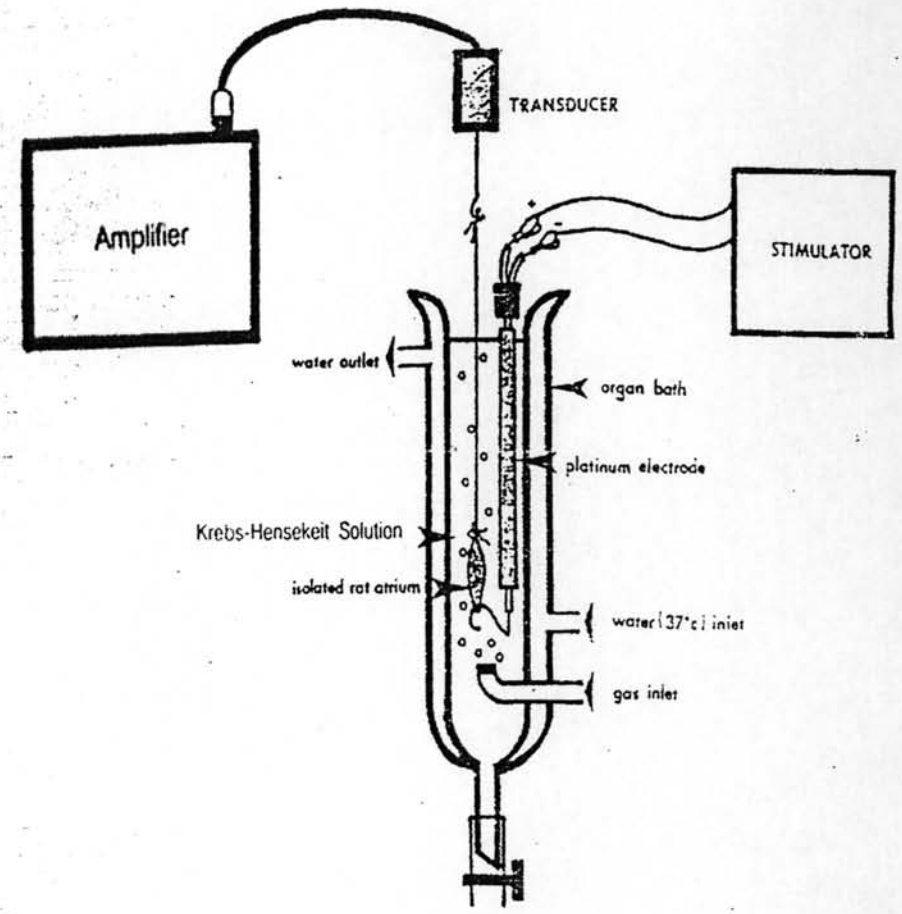

Figure 7 Preparation of isolated rat atria

4.2 Determination of chronotropic and inotropic effects of *Passiflora foetida* extracts on rat isolated atria

The right and left atria were prepared and incubated in Krebs-Henseleit solution until the tension stable. The final concentration of DMSO is 0.25% v/v, which had no significant effect on rate and force of contraction. The PF extracts were cumulatively added to the organ bath at the concentration range of 0.1-100 μg/ml.

The force of contraction of the right atria were measured using isometric force transducer (model MLT050/A, AD Instrument, Castle Hill, Australia). The force of contraction of the left atria was determined using isometric force transducer under the electrical stimulation at 4.2 Hz, impulses of 5 ms duration with threshold at 5 V. The force of contraction of right and left atria were expressed in mg of the developed tension. The heart rate of right atria was expressed in beats per min. The basal contractile responses and heart rate were referred as 100%. The responses were calculated as a change in percentage of contraction or heart rate from the basal responses.

4.3 Investigation of the mechanisms of action of *Passiflora foetida* extracts on rat isolated atria

4.3.1 Effects of *Passiflora foetida* extracts on the activation of β-adrenergic and serotonin receptors.

The chronotropic and inotropic effects of *Passiflora foetida* were further investigated for underlying mechanisms. In order to investigate the involvement of β-

adrenergic receptor and serotonin receptor, 10 μM propranolol HCl (β-adrenergic receptor antagonist), 10 μM ketanserin (5-HT_{2A} receptor antagonist), or both compounds were added to the organ bath 5 min prior to addition of *P. foetida* extracts. The responses were calculated as percent changes of contraction or heart rate from the basal activities.

4.3.2 Effects of *Passiflora foetida* extracts on Ca^{2+} release from internal storage

This experiment procedure was designed to investigate the inotropic effects of *P. foetida* on the basal contraction of isolated rat left atria. The resting state was induced by the continuous "on-off" electrical stimulation. The electrical stimulation started at pacing 4.2 Hz, 5 ms, and 5 V, then stopped for a short periods ranging from 10 seconds to 5 minutes. The effects of *Passiflora foetida* extracts (PF extracts) were tested by incubation with the tissues 5 minute before electrical stimulation as above mentioned. In this study, 10 mM caffeine was also used as positive control because of its known activity to release Ca^{2+} from SR. The initial contractile response of PF extracts on resumption of stimulation after 10 seconds to 5 minutes of rest period (Ti) was compared with the steady-state tension response (Tss) (Yamato et al., 1996) (Figure 8).

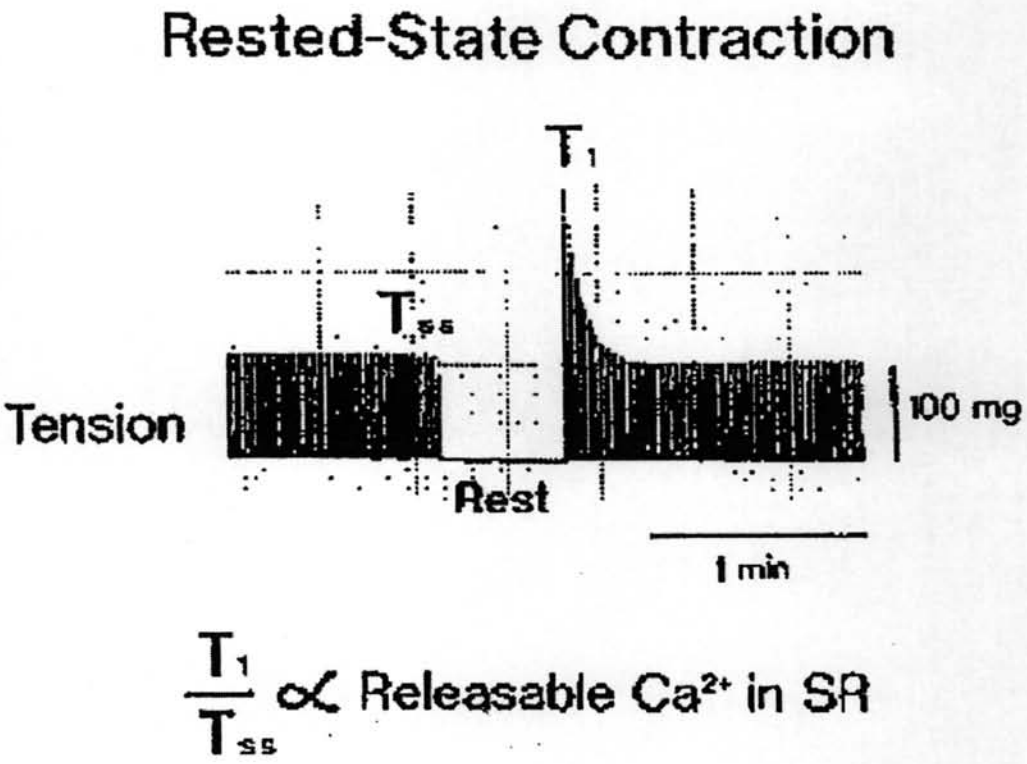

Figure 8 An example of rest-state contraction in papillary muscle. The muscle preparation was stimulated at a fixed stimulation for a certain period and the steady state tension (Tss) was recorded. After stopped stimuation, an initial tension (Ti) was measured as RSC. Ti / Tss was used as an index of releasable Ca^{2+} in sarcoplasmic reticulum (SR) (Yamato et al, 1996).

4.3.3 Effects of *Passiflora foetida* extracts on storage catecholamine

This study was performed in atria isolated from reserpinized rats (5 mg/kg, i.p. for 2 days) in order to investigate the effects of PF extracts on storage catecholamine. Treatment of reserpine induced the depletion of catecholamine. Consequently, tyramine caused positive chronotropic and inotropic effects by releasing storage catecholamine from adrenergic nerve ending. Treatment of tyramine on reserpinized atria resulted in the lack of catecholamine. After atria preparation, 10 μM tyramine was added to confirm a lack of NE in the adrenergic nerve ending. The success of reserpine treatment was elicited by the absence of chronotropic or inotropic effects upon addition of tyramine. Then, the direct effect of PF extract on rate and force of contraction was determined by addition of PF extracts to the reserpinized tissue which had no response toward tyramine. The responses were calculated as a percent change of heart rate of the atria preparation in the absence of PF extracts.

4.3.4 Effects of *Passiflora foetida* extracts on NE and serotonin reuptake

This study was designed to investigate the involvement of PF extract to NE and serotonin reuptake. Reuptake of NE was inhibited by TCAs such as amitriptyline. NE which had been reuptake into noradrenergic storage was blocked and the amount of NE in the synapse was increased, resulted of increasing of force of contraction and heart rate. In addition, reuptake of serotonin was inhibited by fluoxetine, a SSRI and resulted in serotonin increase in synapse. In this study, the following drugs, 10 μM amitriptyline and 1 μM fluoxetine were added to the organ bath 5 min before addition of 0.1 μM NE

and 1 μM serotonin, respectively. Next, PF extract was added to the organ bath 5 min prior to addition of 0.1 μM NE and 1 μM serotonin. The responses were calculated as percent changes of contraction or heart rate from the basal activities.

Data analysis

The results were expressed as means ± S.E.M. Statistical significance of differences were determined by Student's t-test for paired and unpaired data where appropriate. Probability value (P-value) of less than 0.05 was considered to be significant.