CHAPTER 2

MATERIALS AND METHODS

2.1 Collection of *Gracilaria fisheri*

The fresh red marine alga, *G. fisheri* Xia & Abbott was collected from Pattani bay, Pattani province, Thailand, and was authenticated by Associate Professor Yuwadee Peerapornpisal (Applied Algal Research Laboratory, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai).

2.2 Preparation of the aqueous extract of *Gracilaria fisheri*

The fresh *G. fisheri* was washed thoroughly in tab water and dried in an oven at 50°C for 48 h. The dried alga at a weight of 200 g was boiled with 1 L of distilled water at 50°C for 24 h and then filtered through Whatman's filter paper No.4. The filtrate was evaporated and lyophilized to obtain a dry extract. The yield of the aqueous extract of *G. fisheri* (Aq. *G*) was 24.5%. The Aq. *G* extract was dissolved in deionized water before used.

2.3 Evaluation of the Aq. *G* for an antioxidant activity

2.3.1 $ABTS^{+\bullet}$ (2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid) cation radical scavenging activity

The method described by Re *et al.* (1999) (86) with some modifications was used (Fig. 4). The ABTS reagent was prepared by mixing 5 mL of 7 mM ABTS with 88 µL of 140 mM $K_2S_2O_8$. After the mixture was kept in the dark at room temperature for 16 h to allow the completion of radical generation, it was diluted with deionized water to give an absorbance of 0.70 ± 0.05 at 734 nm before use. To determine the scavenging activity, 1 mL ABTS reagent was mixed with 10 µL of sample solution and the absorbance was measured at 734 nm 6 min after the initial

mixing. The $ABTS^{+\bullet}$ radical scavenging activity of the sample was calculated by the following equation:

$$ABTS^{+\bullet} \text{ radical scavenging activity } (\%) = 100 \text{ x } \frac{A_c - A_s}{A_c}$$

where A_c is the absorbance of the control and A_s is the absorbance in presence of sample or positive control.

All determinations were carried out in triplicate. Trolox, a derivative of vitamin E, was employed as a positive control. EC_{50} value (mg/mL) was the effective concentration at which $ABTS^{+\bullet}$ radical was scavenging by 50% and was calculated from a concentration-response curve. The antioxidant activity was expressed as Trolox equivalent antioxidant capacity (TEAC) which represented the concentration (mM) of Trolox per milligram of sample.

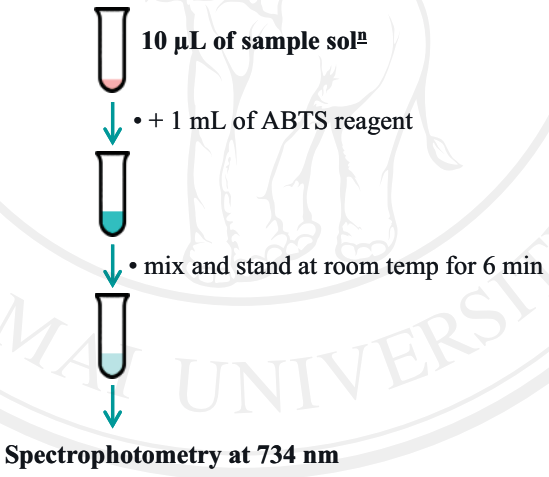

Figure 4 Diagram illustrated the procedure of $ABTS^{+\bullet}$ scavenging activity

2.3.2 Superoxide anion ($O_2^{-\bullet}$) scavenging activity

Measurement of superoxide anion scavenging activity of the Aq. *G* was performed by following the method described by Nishimiki *et al*. (1972) (87) (Fig. 5). The nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH) and phenazine methosulphate (PMS) solutions at the concentrations of 156 µM, 468 µM and 60 µM, respectively were prepared in 0.1 M phosphate buffer (pH 7.4). One mL of NBT solution, 1 mL of NADH solution, and 0.1 mL of sample solution were mixed. The reaction was then started by adding 0.1 mL of PMS solution to the mixture. After

5 min of incubation at room temperature, the absorbance was measured at 560 nm and the scavenging activity on superoxide anion was calculated by the following equation:

$$\text{Superoxide anion radical scavenging activity (\%)} = 100 \text{ x } \frac{A_c - A_s}{A_c}$$

where A_c is the absorbance of the control and A_s is the absorbance in presence of sample or positive control.

All determinations were carried out in triplicate. Gallic acid was used as a positive control. The EC_{50} value (mg/mL) which stands for the concentration required for 50% superoxide anion radical scavenging activity were calculated from concentration-response curve. The superoxide anion scavenging activity of samples was expressed as gallic acid equivalent (GAE) in gram sample per milligram of gallic acid.

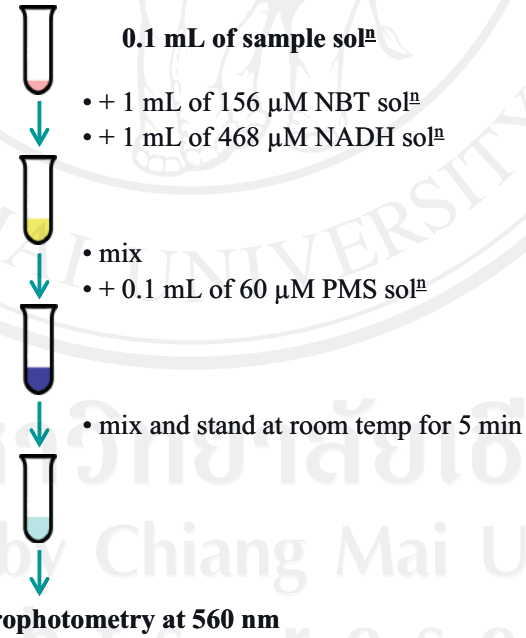

Figure 5 Diagram illustrated the procedure of superoxide anion scavenging activity

2.3.3 Hydroxyl radical ($^{\bullet}OH$) scavenging activity

Scavenging of hydroxyl radicals ($^{\bullet}OH$) was determined by the method of Halliwell *et al*. (1987) (88) (Fig. 6). Solutions of 2.8 mM deoxyribose, 2.8 mM H_2O_2,

25 μM $FeCl_3$, 100 μM nitrilotriacetic acid (NTA), 1% (w/v) thiobarbituric acid (TBA) and 2.8% (w/v) trichlorotic acid (TCA) were prepared in 0.1 M phosphate buffer (pH 7.4). The reaction mixtures contained, in a final volume of 1.2 mL, the following reagents: 0.22 mL of sample solution, 0.2 mL of deoxyribose solution, 0.38 mL of H_2O_2 solution, 0.2 mL of $FeCl_3$ solution, and 0.2 mL of NTA solution. The mixtures were incubated in a water bath at 37°c for 60 min. Degradation of deoxyribose sugar induced by $^{\bullet}OH$ was determined by the addition of 1 mL of TBA solution and 1 mL of TCA solution and heated at 100°c for 20 min. The pink chromogen formed was determined by measuring its absorbance at 532 nm. The scavenging activity on hydroxyl radicals (inhibition of deoxyribose degradation) was calculated by the following equation:

$$\text{Hydroxyl radical scavenging activity (\%)} = 100 \text{ x } \frac{A_C - A_s}{A_c}$$

where A_c is the absorbance of the control and A_s is the absorbance in presence of sample or positive control.

All determinations were carried out in triplicate. Trolox was used as a positive control. EC_{50} value (mg/mL) was the effective concentration at which hydroxyl radical was scavenging by 50% and was calculated from a concentration-response curve. The hydroxyl radical scavenging activity of samples was expressed as Trolox equivalent antioxidant capacity (TEAC).

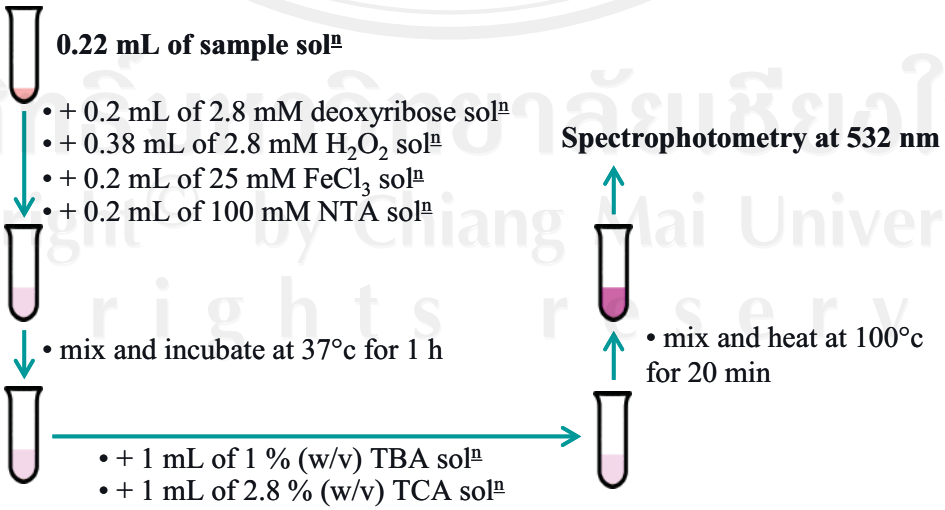

Figure 6 Diagram illustrated the procedure of hydroxyl radical scavenging activity

2.3.4 Inhibition of lipid peroxidation

The lipid peroxide formation was measured by the method of Masao *et al.* (1993) (89) (Fig. 7). The rats weighing 200-250 g were sacrificed by dislocation of their necks. The abdomen was opened, and the liver was removed. The liver was then homogenized in 150 mM Tris-HCl buffer (pH 7.2). Total protein concentration was estimated by the method of Lowry *et al.* (90) using bovine serum albumin (BSA) as a standard. The reaction mixture was prepared composing of 0.2 mL of rat liver homogenate in 150 mM Tris-HCl buffer (pH 7.2), 0.5 mM ferrous chloride solution, 0.06 mM ascorbic acid solution, and sample solution in a final volume of 1.0 mL. The mixture was then incubated at 37°c for 1 h. The incubated reaction mixture (0.4 mL) was mixed with 0.2 mL of 0.8% (w/v) thiobarbituric acid, 1.5 mL of 20% acetic acid and 0.4 mL of deionized water. Then, the mixture was heated in a water bath at 100°c for 1 h. After the mixture was cooled, 1 mL of deionized water and 5 mL of *n*-butanol were added, followed by vigorously shaking for 1 min. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured. The inhibition against formation of lipid peroxide of rat liver was calculated by the following equation:

$$\text{Inhibition of lipid peroxide (\%)} = 100 \text{ x } \frac{A_c - A_s}{A_c}$$

where A_c is the absorbance of the control and A_s is the absorbance in presence of sample or positive control.

All determinations were carried out in triplicate. Trolox was used as a positive control. EC_{50} value (mg/mL) was the effective concentration at which lipid peroxide was inhibiting by 50% and was calculated from a concentration-response curve. Inhibition of lipid peroxidation was expressed as Trolox equivalent antioxidant capacity (TEAC).

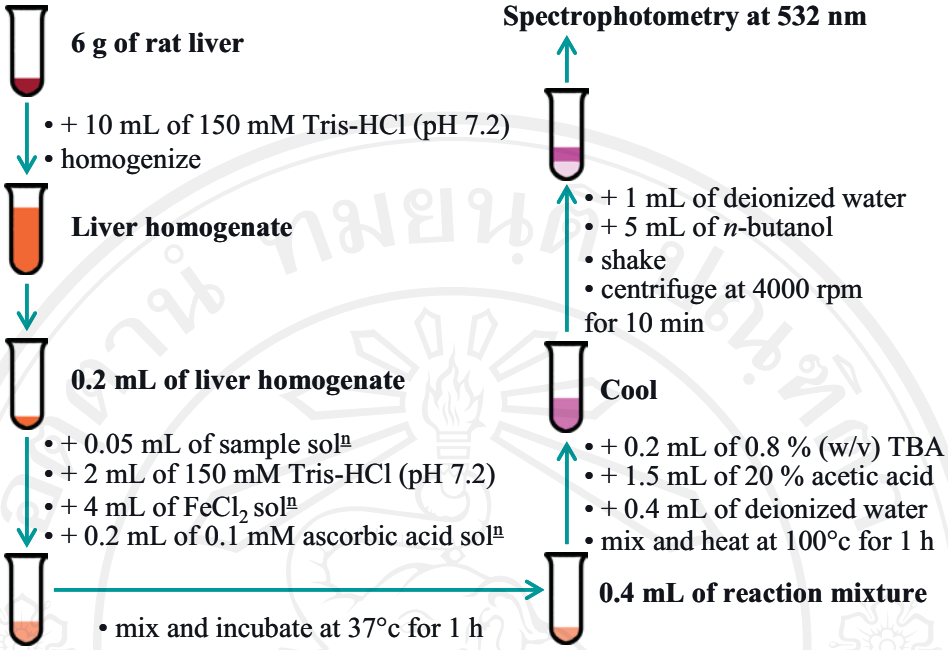

Figure 7 Diagram illustrated the procedure of inhibition of lipid peroxidation

2.3.5 Metal chelating activity

The chelating of ferrous ions (Fe^{2+}) by Aq. *G* was estimated by the Ferrozine assay (91) (Fig. 8). Briefly, 50 µL of 2 mM ferrous chloride solution was added to a 0.4 mL of sample solution. The reaction was initiated by the addition of 5 mM ferrozine solution (0.2 mL). Then, the mixture was shaken vigorously and left at room temperature for 10 min, and was then measured spectrophotometrically at 562 nm. The metal chelating activity was calculated by the following equation:

$$\text{Metal chelating activity (\%)} = 100 \times \frac{A_c - A_s}{A_c}$$

where A_c is the absorbance of the control and A_s is the absorbance in presence of sample or positive control.

All determinations were carried out in triplicate. EDTA, strong chelators, was used as a positive control. EC_{50} value (mg/mL) was the concentration at which metal was chelating by 50% and was calculated from a concentration-response curve. The metal chelating activity of samples was expressed as EDTA equivalent (EDTAE) in microgram EDTA per gram of sample.

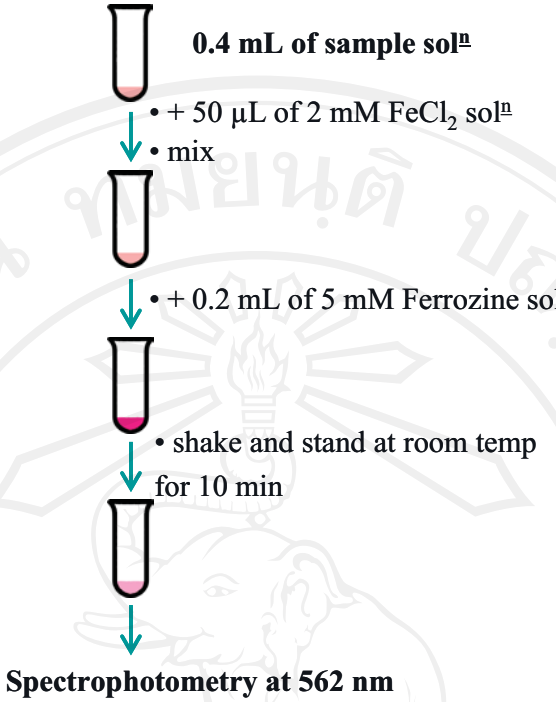

Figure 8 Diagram illustrated the procedure of metal chelating activity

2.3.6 Reducing power

The determination of the reducing power was conducted according to the method of Oyaizu (1986) (92) (Fig. 9). A 2.5 mL of sample solution was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide solution, after which the reaction mixture was incubated in a water bath at 50°c for 20 min. A 2.5 mL of 10% (w/v) trichloroacetic acid solution was added to the mixture and centrifuged at 3000 rpm for 10 min. The supernatant of solution (2.5 mL) was mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% of ferric chloride solution. The absorbance was measured spectrophotometrically at 700 nm. A higher absorbance of the reaction mixture represents stronger reducing power.

All determinations were carried out in triplicate. Gallic acid was used as a positive control. The reducing power of samples was expressed as gallic acid equivalent (GAE).

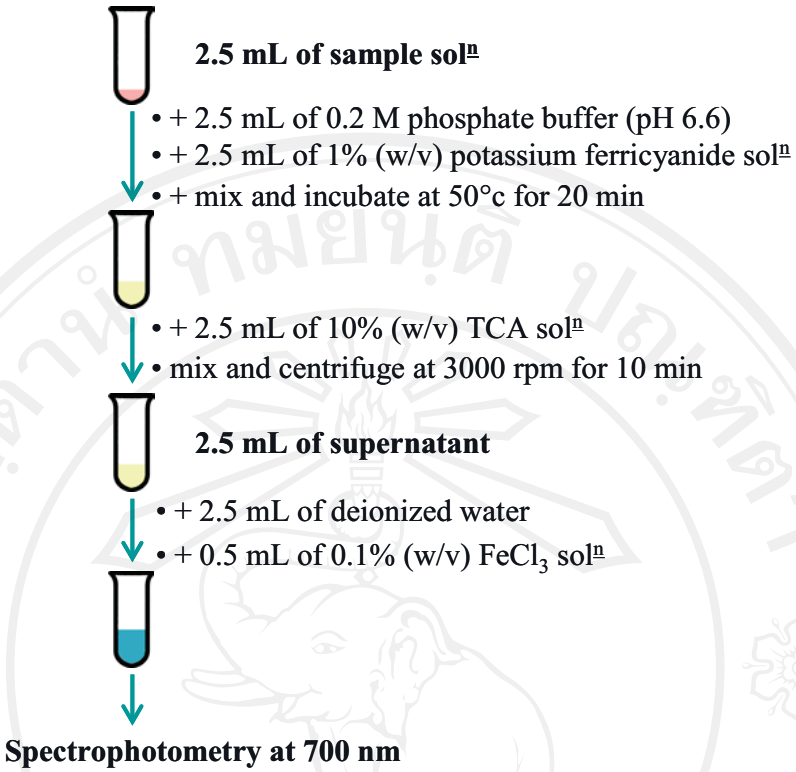

Figure 9 Diagram illustrated the procedure of reducing power

2.4 Determination of phenolic content of *G.fisheri*

The phenolic content in the Aq. *G* was determined according to the method of Hammerschmidt and Pratt (1978) (93) with minor modifications (Fig. 10). Briefly, 0.2 mL of the sample solution was mixed with 1.0 mL of 10% Folin-Ciocalteu solution and 0.8 mL of 7.5% sodium carbonate solution. The mixture was allowed to stand for 1 h at room temperature, and the absorbance was then measured at 765 nm. Gallic acid was used as the standard for the calibration curve, and the total phenolic content was expressed as GAE in gram of tested extract per milligram gallic acid.

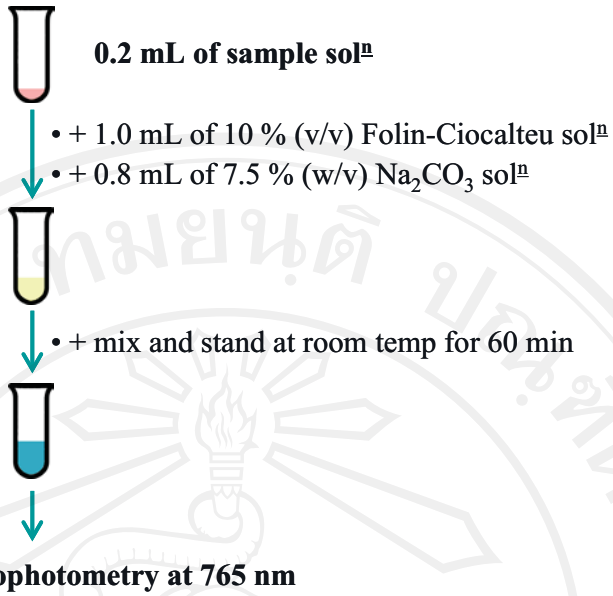

Figure 10 Diagram illustrated the procedure of determination of total phenolic content

2.5 Determination of cardiovascular activity

2.5.1 Laboratory animals

Male Sprague-Dawley rats weighing 200-250 g were purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Thailand. They were kept in an animal room where the temperature was maintained at 22 ± 3°C with a 12 h light-dark cycle. The animals had free access to water and food (Perfect Companion, Bangkok, Thailand); were acclimatized for at least one week before starting the experiments. The experimental procedures were approved by the Animal Ethics Committee, Faculty of Medicine, and Chiang Mai University, Thailand.

2.5.2 Blood pressure and heart rate of thiopental anesthetized hypertensive rats

The experiment was modified as described by Vogel and Vogel (1997) (94) (Fig. 11). Rats were anesthetized by an intraperitoneal injection of thiopental (90 mg/kg). A tracheotomy was performed and a polyethylene tube was inserted into the trachea for artificial respiration. The Aq. *G* was slowly injected via a cannula inserted into the external jugular vein. Blood pressure was recorded from the common carotid

artery via an arterial cannula connected to a pressure transducer (Gould P231D, Statham Instruments, U.S.A.) and coupled with a Grass polygraph (Model 7D; Grass Instrument Co., U.S.A.). Heart rate was monitored by a tachograph (Model 7DA; Grass Instrument Co., U.S.A.).

Blood pressure was expressed as the mean arterial blood pressure (MABP) which was calculated according to the following equation.

$$MABP = P_d + \frac{P_s - P_d}{3}$$

where Ps = Systolic blood pressure

Pd = Diastolic blood pressure

N^{ω}-Nitro-L-arginine methyl ester (L-NAME; a nitric oxide synthase inhibitor) at the dose of 3 mg/kg was used to induce hypertension (95). Increased blood pressure was allowed to equilibrate at least for 15 min before commencing the experiment.

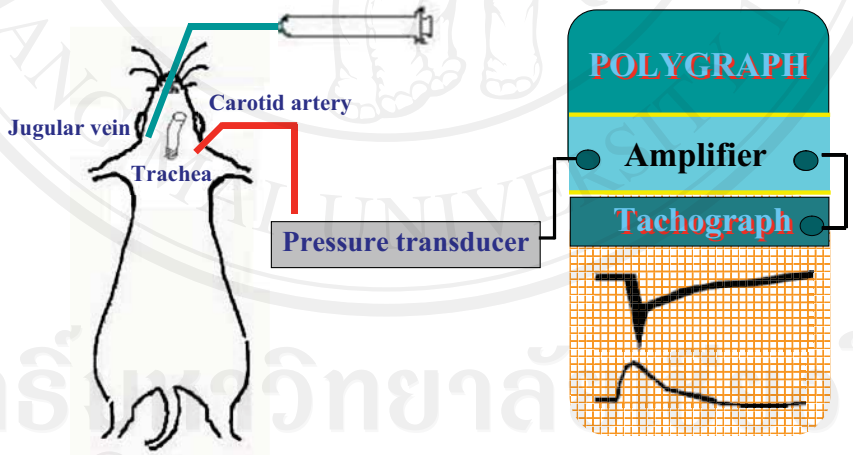

Figure 11 Diagram illustrated the set up of Preparation of the experimental animals for recording blood pressure and heart rate

2.5.3 Isolated rat atria preparation

The preparation was performed as described in the book "Pharmacological Experiments on Isolated Preparations" (96) (Fig. 12). The rat was sacrificed by a cervical dislocation. The thorax was opened; the heart was carefully dissected out and

placed in Feigen's solution (NaCl = 0.9 g/L, $NaHCO_3$, = 0.6 g/L, KCl = 0.42 g/L, $CaCl_2$ = 0.62 g/L and glucose = 1.0 g/L) bubbled with 100% O_2 and 5% CO_2 at room temperature. The right atria was carefully separated from the rest of the heart and mounted in an organ bath containing Feigen's solution at 37°c and aerated continuously with 100% O_2 and 5% CO_2. Isometric contraction of the atria was recorded via a force displacement transducer (FT03, Grass Instrument Co., U.S.A.) and heart rate was recorded via a tachograph connected to a Grass polygraph. The tension of 1 g was applied to the atria.

A 30 minute equilibration period was allowed before starting the experiment. The effect of the Aq. *G* on force and rate of contraction was observed.

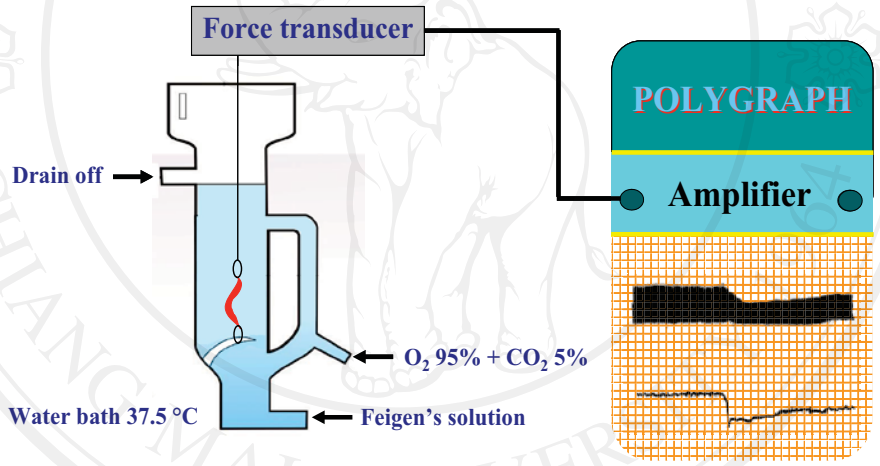

Figure 12 Diagram illustrated the set up of the isolated rat right atria experiment

2.5.4 Isolated rat aorta preparation

The preparation was prepared according to the method of Hodoglugil *et al.* (97) (Fig. 13). The rat was sacrificed by a cervical dislocation. The thoracic aorta was excised immediately and immersed in Kreb's solution (NaCl = 6.9 g/L, $NaHCO_3$, = 2.1 g/L, KCl = 0.35 g/L, $MgSO_4.7H_2O$ = 0.29 g/L, KH_2PO_4 = 0.16 g/L, $CaCl_2.2H_2O$ = 0.25 g/L and glucose = 2.0 g/L) aerated with 100% O_2 and 5% CO_2 at room temperature. After cleaning the aorta free of fat and other adhering tissues, the vessel was cut about 3 mm length with special care being taken to avoid damaging the luminal surface for endothelium intact preparation. Denuded aortic rings were prepared by gently rubbing the inner surface with a roughened stainless steel rod. The

endothelium intact ring or endothelium denuded ring preparation was mounted in a tissue chamber containing Kreb's solution at 37°c and aerated continuously with 95% O_2 and 5% CO_2. The tension of 1 g was applied and isometric contraction was recorded by means of a polygraph (Model 7D; Grass Instrument Co., U.S.A.) via a force displacement transducer (FT03, Grass Instrument Co., U.S.A.). The aortic ring preparation was left to equilibrate for 1 h. The endothelial function was assessed by using acetylcholine (20µg/mL). Vasorelaxant activity of the Aq. *G* was tested in endothelium intact and endothelium denuded aorta. The contraction of the rings was induced by high K^+ (60 mM) or phenylephrine (10 µM). Vasorelaxant activity of the Aq. *G* was assessed by cumulative administration of the extract after induced contractions have reached the peak level.

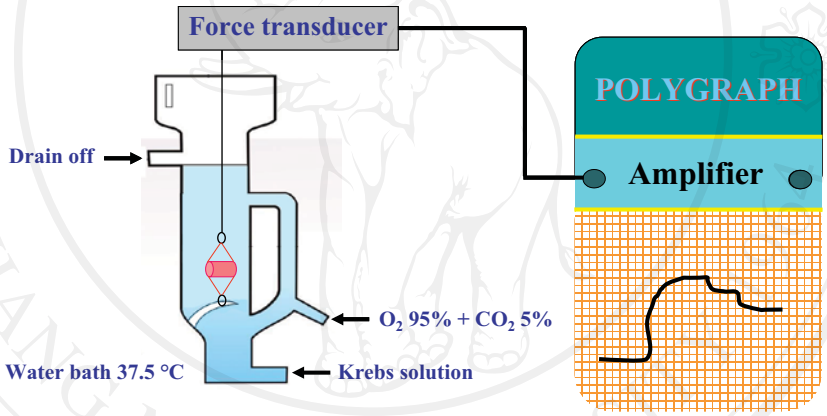

Figure 13 Diagram illustrated the set up of isolated rat aorta experiment

2.6 Statistical analysis

The data were expressed as mean ± S.E.M. Statistical comparison between groups was analyzed by using one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test. Statistical comparison between before and after treatments was performed by using Student's t-test. *P* values less than 0.05 were considered significant.

2.7 Drugs and chemicals

Drugs

1. Acetylcholine iodide (Sigma-Aldrich, Steinheim, Germany)

2. N^{ω}-Nitro-L-arginine methyl ester: L-NAME (Sigma-Aldrich, Steinheim, Germany)
3. Phenylephrine (Sigma-Aldrich, Steinheim, Germany)
4. Thiopental (Research Institute of Antibiotics and Bio-transformations, Czech Republic)

Chemicals

1. Bovine serum albumin (BSA) (Invitrogen ^{TM}, Germany)
2. 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Sigma-Aldrich, Steinheim, Germany)
3. Potassium peroxodisulfate (Sigma-Aldrich, Steinheim, Germany)
4. Nitro tetrazolium blue (Sigma-Aldrich, Steinheim, Germany)
5. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Sigma-Aldrich, Steinheim, Germany)
6. β-Nicotinamide adenine dinucleotide (Sigma-Aldrich, Steinheim, Germany)
7. Phenazine methosulphate (Sigma-Aldrich, Steinheim, Germany)
8. Gallic acid (Sigma-Aldrich, Steinheim, Germany)
9. 2-Deoxy-D-ribose (Sigma-Aldrich, Steinheim, Germany)
10. Hydrogen peroxide (Merck, Darmstadt, Germany)
11. Ferric chloride (Sigma-Aldrich, Steinheim, Germany)
12. Nitrilotriacetic acid (Sigma-Aldrich, Steinheim, Germany)
13. 2-Thiobarbituric acid (Fluka, Switzerland)
14. Trichlorotic acid (Sigma-Aldrich, Steinheim, Germany)
15. Tris-HCl (Merck, Darmstadt, Germany)
16. Ferrous chloride tetrahydrate (Sigma-Aldrich, Steinheim, Germany)
17. Ascorbic acid (Sigma-Aldrich, Steinheim, Germany)
18. *n*-Butanol (Lab Scan Asia CO., Bangkok, Thailand)
19. Ferrozine (Sigma-Aldrich, Steinheim, Germany)
20. Potassium ferricyanide (Sigma-Aldrich, Steinheim, Germany)
21. Ethylenediaminetetraacetic acid (Sigma-Aldrich, Steinheim, Germany)
22. Folin-Ciocalteu's phenol reagent (Merck, Darmstadt, Germany)
23. Sodium carbonate (Sigma-Aldrich, Steinheim, Germany)

24. Calcium chloride (Riedel-De Haen Ag Seelze-Hannover)
25. Glucose (May & Baker Ltd., Dagenham, England)
26. Magnesium sulphate (A Searl Company, England)
27. Potassium chloride (May & Baker Ltd., Dagenham, England)
28. Potassium dihydrogen phosphate (Merck, Darmstadt, Germany)
29. Sodium bicarbonate (Vidhyasom Co Ltd., Bangkok, Thailand)
30. Sodium chloride (Merck, Darmstadt, Germany)