CHAPTER 2

MATERIALS AND METHODS

Preparation of an aqueous extract of *Caulerpa racemosa* var. *cylindracea*

The green seaweed: *C. racemosa* var. *cylindracea* (Family Caulerpaceae) was collected at Klongyang, Krabi province, Thailand and identified by Assistant Professor Mantana Nualchareon (Department of Biology, Faculty of Science and Technology, Rajabhat Phuket University, Phuket). The seaweed was then authenticated by Associate Professor Yuwadee Peerapornpisal (Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai). The specimen of *C. racemosa* was deposited at the herbarium of Rajabhat University, and at the Applied Algal Research Laboratory, Department of Biology, Faculty of Science, Chiang Mai University.

The seaweed was cleaned with fresh water and then dried in the shade for 24-48 h. The dried *C. racemosa* was macerated in distilled water and warmed in a water bath at 50°C for 24 h. The mixture was filtered through Whatman no.4 filter paper. The filtrate obtained was concentrated under a reduced pressure at 60°C by a rotary evaporator, and freeze-dried using a lyophilizer. The aqueous extract obtained was dissolved in distilled water before using

Experimental animals

Male Sprague-Dawley rats weighing 200-250 g were purchased from The National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand**.** The animals were kept in an animal room, Faculty of Medicine, Chiang Mai University where the temperature was maintained at 22±2°C under a 12 h light-dark cycle. All animals were fed with standard diet (Perfect Companion, Bangkok, Thailand) and water *ad libitum.*

Experiment protocols

1. Anti-gastric ulcer activity

The aqueous extract of *C. racemosa* was tested for an anti-ulcer activity in rats, using experimental models including: (1) restraint water immersion stress- (2) HCl/Ethanol- and (3) Indomethacin-induced gastric ulcers.

The rats were divided into 4 groups of 6 rats as follows:

Group 1 control group, the rats were orally administered distilled water (vehicle)

Group 2 reference group, the rats were orally administered cimetidine at the dose of 100 mg/kg

Group 3, 4 test groups, the rats were orally administered the AqCR at doses of 100 and 500 mg/kg, respectively.

The rats were fasted 48 h, and water was given *ad libitum*. The water was withdrawn 1 h before starting the experiment. Cimetidine, the AqCR or vehicle (distilled water) was given orally 1 h before induction of gastric ulcers.

1.1) Restraint water immersion stress-induced gastric ulcers

The method described by Takagi *et al.* (1963) (93) was used. The rats were placed individually in stainless steel cages and immersed up to the level of the xiphoid in a water-bath maintained at 22±2°C. The rats were sacrificed 5 h later for determination of gastric ulcers. The protocol is shown in Figure 6.

1.2) HCl/Ethanol-induced gastric ulcers

The experiment was performed according to the method of Mizui and Deteuchi (1988) (94) and the protocol is depicted in Figure 7. Each rat was administered 1 ml of HCl/Ethanol (60 ml absolute ethanol + 1.7 ml HCl 36.5% + 38.3 ml distilled water) orally. One hour later, the rats were sacrificed and determined of gastric ulcers.

1.3) Indomethacin-induced gastric ulcers

The procedure of Morimoto *et al.* (1991) (95) was employed. The rats were given indomethacin (suspended in 0.5% tween 80) at the dose 30 mg/kg by an intraperitoneal injection, and they were sacrificed 5 h later for determination of gastric ulcers. Figure 8 shown the protocol of the experiment.

Evaluation of gastric ulcers

The stomach was opened along the greater curvature, rinsed with isotonic saline and pinned out on a wax plate. The glandular portion of the stomach was then examined for ulcers. The length (mm) of each ulcer was measured under a dissecting microscope (10X). The ulcer index and percent inhibition of gastric ulcer was determined as follow:

$$\text{Ulcer index (UI)} = \frac{\text{Sum of the total length of lesions in each group}}{\text{Number of rats in the group}}$$

$$\% \text{ Inhibition} = \left(\frac{UI_c - UI_t}{UI_c} \right) X100$$

Where

UI_c = Ulcer index of contol group

UI_t = Ulcer index of test group

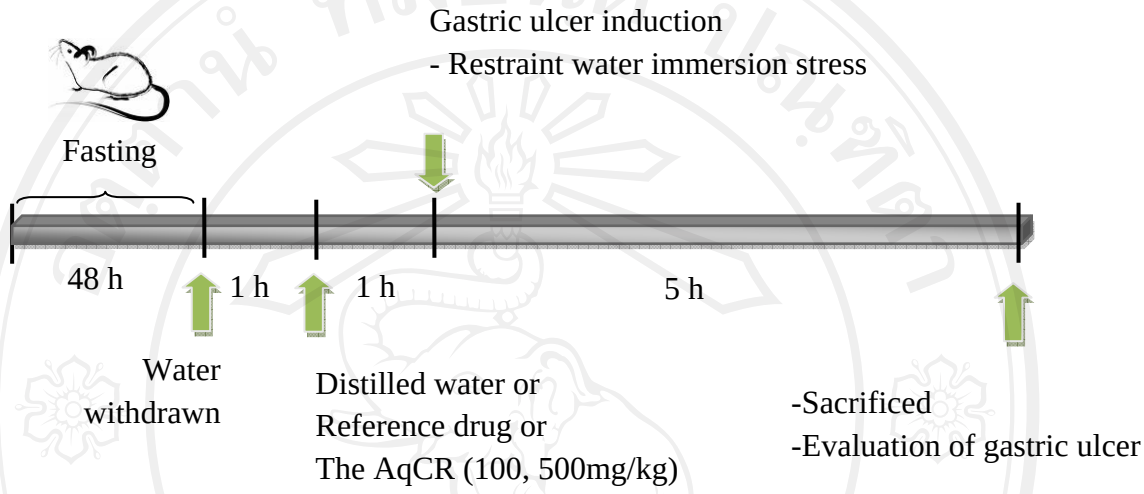

Figure 6 Diagram illustrated the protocol of anti-gastric ulcer test: restraint water immersion stress-induced gastric ulcers in rats

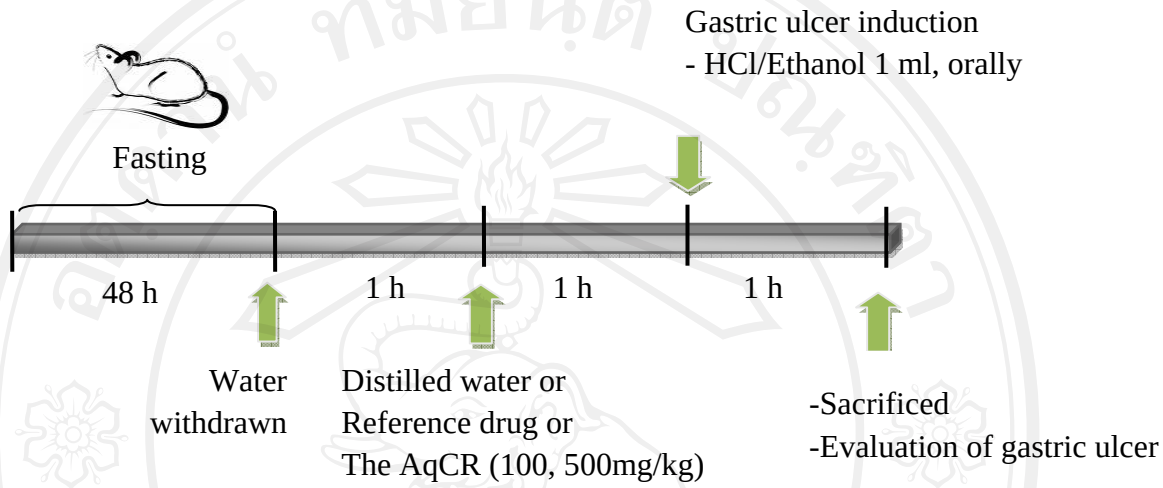

Figure 7 Diagram illustrated the protocol of anti-gastric ulcer test: HCl/Ethanol-induced gastric ulcers in rats

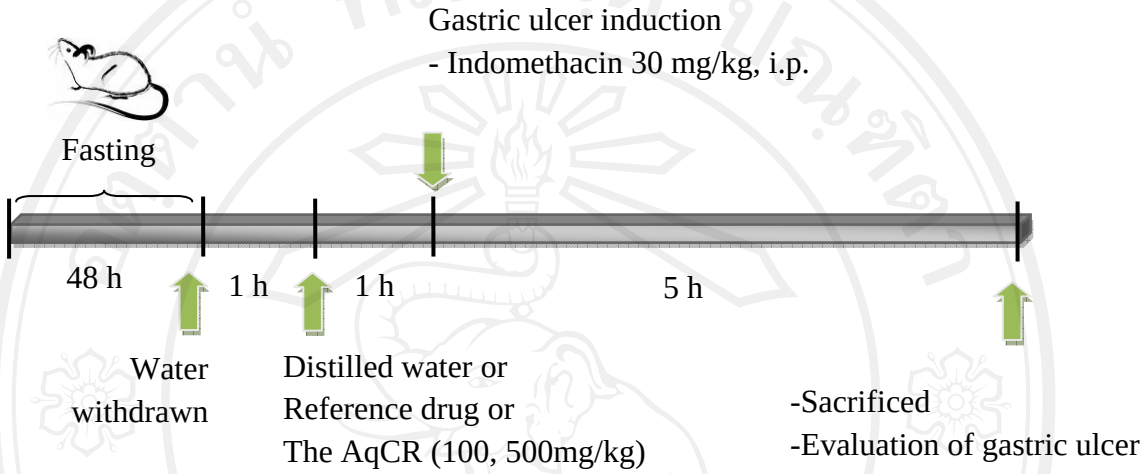

Figure 8 Diagram illustrated the protocol of anti-gastric ulcer test: Indomethacin - induced gastric ulcers in rats

2. Hepatoprotective activity

The rats were divided into 6 groups of six rats each: Figure 9 illustrates the protocol of the test for hepatoprotective activity.

Group 1: normal group. The rats were orally administered distilled water

Group 2: the rats were orally administered the AqCR at a dose of 500 mg/kg for 7 days

Groups 3-6: CCl_4-induced hepatotoxicity. CCl_4 in olive oil (1:1) was subcutaneously injected at a dose of 2 ml/kg on day 7, 1 h after the last administration of distilled water, silymarin or the AqCR as shown below:

Group 3: control group, the rats were orally administered distilled water for 7 days

Group 4: reference group, the rats were orally administered orally silymarin at a dose of 100 mg/kg for 7 days

Group 5, 6: test groups, the rats were orally administered orally the AqCR at the dose of 100 and 500 mg/kg, respectively, for 7 days.

2.1) Collection of blood samples

Blood collection was performed on day 8 of the experiment. Five ml of blood was withdrawn from the common carotid artery of the thiopental anesthetized rats. Serum was then separated by centrifugation of blood samples at 2,500 rpm for 10 min. The serum was sent to the Lanna Medical Laboratory, Chiang Mai for determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels.

2.2) Histopathologic examination of liver

Livers of the rats were fixed in 10% formalin diluted with distilled water and processed for paraffin embedding. Sections were stained with hematoxylin and eosin and histopathologic examination of the sections was performed by Associate. Prof. Dr. Nirush Lertprasertsuk, Department of Pathology, Faculty of Medicine, Chiang Mai University.

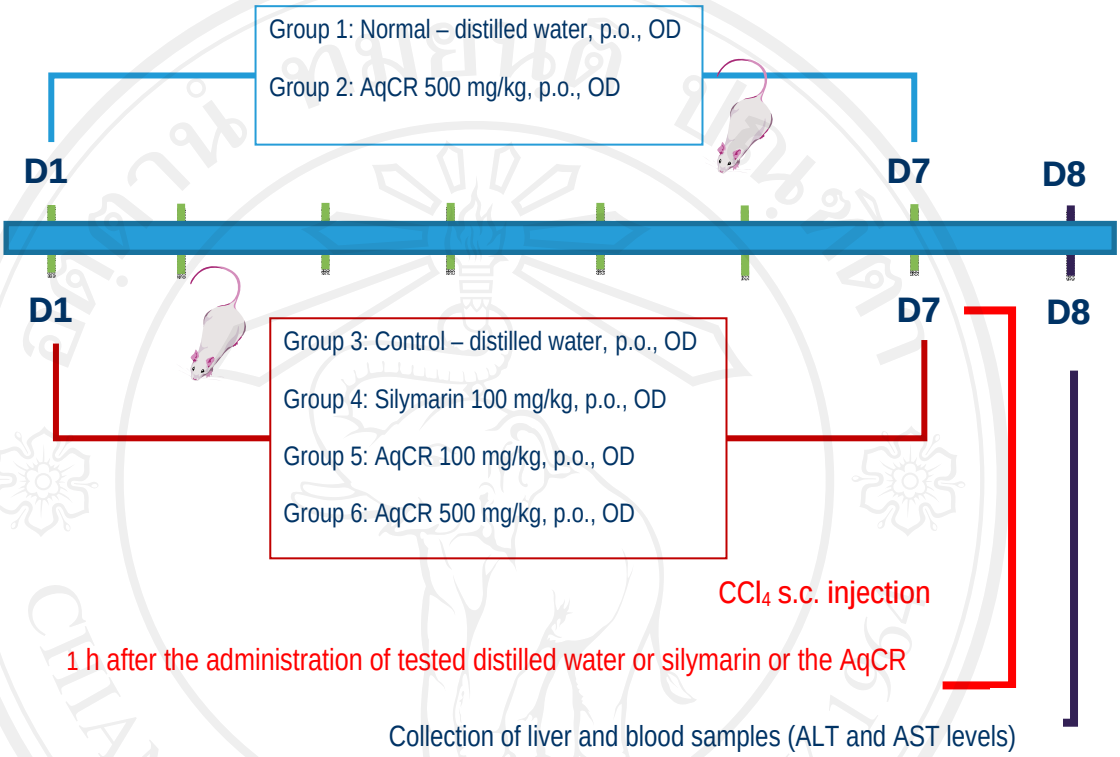

Figure 9 Diagram illustrated the protocol of the test for hepatoprotective activity

3. Antioxidant activity

3.1) 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) assay

The DPPH radical-scavenging effect was measured according to the method of Hou *et al.* (2001) (96). Briefly, each 0.3 ml of the test sample was added to 0.1 ml buffer (pH 7.9), and then mixed with 0.6 ml of 100 mM DPPH in methanol for 20 min under light protection. The absorbance was read at 517 nm by UV-visible spectrophotometer. Deionized water was used as the blank. The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = [(Ab - As)/Ab] \times 100$$

Ab = absorbance at 517 nm of the blank
As = absorbance at 517 nm of the sample

Gallic acid was used as a standard. All determinations were carried out in triplicate. The EC50 which stands for the concentration required for 50% scavenging activity were calculated from concentration- response (scavenging activity) curve. The antioxidant activity of the samples was expressed as gallic acid equivalent (GAE).

3.2) Anti-lipid peroxidation activity

The method used was modified from that of Masao *et al.* (1993) (97). Briefly, 0.5g of the rat liver tissue was sliced and homogenized with 10 ml of 150 mM KCl-Tris-HCl buffer (pH 7.2). The reaction mixtures composed of 0.25 ml of liver homogenate; 2 ml of Tris-HCl buffer (pH 7.2), 0.2 ml of 0.1 mM ascorbic acid, 4 ml $FeCl_2$ and 0.05 ml of various concentrations of aqueous extract of *C. racemosa* were prepared. The mixtures were incubated at 37°C for 1 h in capped tubes. Then, 0.1 N HCl , 0.2 ml of 9.8% sodium dodecyl sulphate, 0.9 ml of distilled water and 2 ml of 0.6% thiobarbituric acid (TBA) were added to each tube and vigorously shaken. The tubes were placed in a water bath at 80°C for 30 min, and then cooled. The mixtures were precipitated by adding 5 ml of *n*-butanol. The flocculant precipitate was separated by centrifugation at 3,000 rpm for 10 min. The absorbance of the obtained supernatant was determined at 532 nm by a UV-visible spectrophotometer. Figure 10 illustrates the protocol of the experiment.

Inhibition of lipid peroxidation (%) = [(Ab -As)/Ab] x100

Ab = absorbance at 532 nm of the blank

As = absorbance at 532 nm of the sample

All determinations were carried out in triplicate. The EC50 was calculated from concentration-response (inhibition of lipid peroxidation) curve. The inhibition was expressed as Trolox equivalent antioxidant capacity (TEAC).

Total protein concentration was estimated by the method of Lowry *et al.* (1951) (98) using bovine serum albumin (BSA) as a standard.

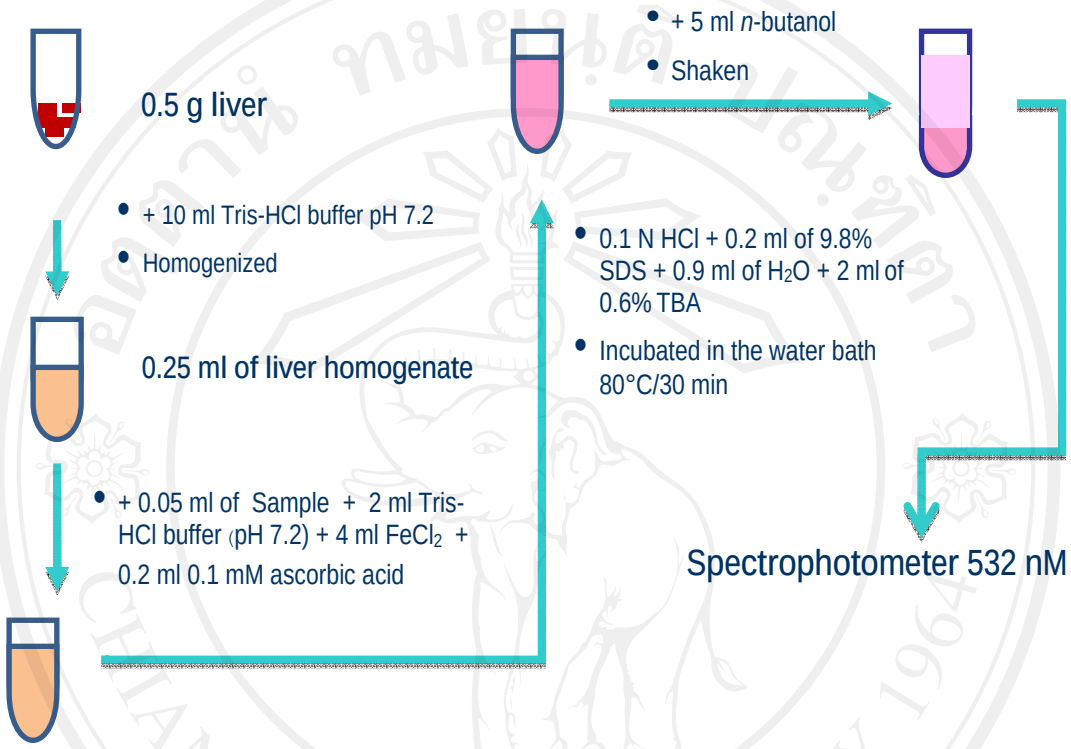

Figure 10 Diagram illustrated the producedure of anti-lipid peroxidation activity

4. Total phenolic contents

The content of phenolic compounds of the aqueous extract of *C. racemosa* was determined by a modification of the Follin-Ciocalteu method (99). 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% Na_2CO_3 solution. The mixture was allowed to stand for 1 h at room temperature, and the absorbance was then measured at 765 nm by a UV-visible spectrophotometer. All determinations were carried out in triplicate. The content of phenolic compounds was expressed as gallic acid equivalent (GAE).

Statitical analysis

The data from the experiments were expressed as mean±standard error of mean (S.E.M.). Statistical comparison between groups were analyzed by using ANOVA and post hoc least-significant difference (LSD) test and *p* values less than 0.05 were considered significant.

Drugs and Chemicals

Drugs

1. Cimetidine (Atlantic, Bangkok, Thailand)
2. Indomethacin (Sigma Chemical Company, St. Louis, U.S.A.)
3. Thiopental (VIDHYASOM CO., Bangkok, Thailand.)

Chemicals

1. Ascorbic acid (Sigma Chemical Company, St. Louis, U.S.A.)
2. Bovine serum albumin (BSA): (Invitrogen ™, Germany)
3. Carbon tetra Chloride: CCl_4 (VIDYASOM CO., Bangkok, Thailand)
4. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) reagent radical (Sigma Chemical Company, St. Louis, U.S.A.)
5. Ethanol (MERCK, Darmstadt, F.R. Germany)
6. Ferous chloride tetrahydrate: (Sigma Chemical Company, St. Louis, U.S.A.)
7. Folin-Ciocalteu's phenol reagent (MERCK, Darmstadt, F.R. Germany)
8. Gallic acid (Sigma Chemical Company, St. Louis, U.S.A.)
9. Hydrochloric acid (BDH Laboratory Spplies Poole, England)
10. Methanol (Lab SCAN ASIA CO., Bangkok, Thailand.)
11. *n*-butanol. (Lab SCAN ASIA CO., Bangkok, Thailand.)
12. Olive oil (VIDHYASOM CO., Bangkok, Thailand.)
13. Silymarin (Sigma Chemical Company, St. Louis, U.S.A.)
14. Sodium Bicarbonate: Na_2CO_3 (Sigma Chemical Company, St. Louis, U.S.A.)
15. Sodium dodecyl sulphate: SDS (Sigma Chemical Company, St. Louis, U.S.A.)
16. Thiobarbituric acid: TBA (Fluka, Switzerland.)
17. Tris-HCl buffer (pH 7.2) (MERCK, Darmstadt, F.R. Germany)
18. Trolox (Sigma Chemical Company, St. Louis, U.S.A.)