CHAPTER III

MATERIALS AND METHODS

3.1 Sample Preparation

The characteristics of ten selected flowers used in this study are shown in Table 4. *Hibiscus rosa-sinensis* Linn. (ชบา), *Antigonon leptopus* Hook. & Arn.(พวงชมพู), *Ixora coccinea* Linn.(เข็ม), *Syzygium malaccense* (Linn.) Merr.& Perry (เศรษฐชมพู่), *Millingtonia hortensis* Linn, (ปีป) *Rhinacanthus nasutus* (Linn.) Kurz. (ทองพันชั่ง), *Plumeria obtusa* Linn (ลั่นทม). were collected from Bangkok, Thailand. *Nelumbo nucifera* Gaertn (บัวหลวง), *Curcuma sessilis* Gage.(กระเจียว), *Punica granatum* Linn.(ทับทิม) were collected from Banphai District, Khon Kaen province, Thailand. They were collected from January to April 2006. Identified by comparing with the herbarium specimens from the office of the forest herbarium and the voucher specimens were deposited in the faculty of Pharmaceutical Science, Department of Food Chemistry, Chulalongkorn University. All kinds of flower were washed with tap water and dried at 40 °C until it was dried. The dried samples were pulverized and kept in a well-sealed, light-resistant container. A 20-g sample of dried powder was extracted sequentially with 200 ml of dichloromethane, methanol and then water under reflux for 3 h. Each step was repeated 3 times (Figure 19). After filtration, each pooled extract was dried in a vacuum rotary evaporator, weighed and dissolved in dimethyl sulfoxide (dichloromethane and methanol extract of flowers) and water (water extract of flowers) for Ames test. However, dichloromethane, methanol and water extract of flowers were dissolved in water for somatic mutation and recombination test.

Table 4. Selected flowers in this study.

Scientific name	Common name	Thai name	Family	Colour of flower
Hibiscus rosa-sinensis Linn	Red hibiscus	Chaba	MALVACEAE	Red
Antigonon leptopus Hook. & Arn.	Mexican creeper	Phuang chomphu	POLYGONACEAE	Pink
Ixora coccinea Linn.	Ixora	Khem daeng	RUBIACEAE	Red
Plumeria obtusa Linn.	White frangipani	Lan thom khao	APOCYNACEAE	White
Syzygium malaccense (Linn.) Merr.& Perry.	Malay apple	Chomphu ma miao	MYRTACEAE	Red
Curcuma sessilis Gage.	-	Kra chiew	ZINGIBERACEAE	Red
Nelumbo nucifera Gaertn.	Sacred lotus	Bua luang	NYMPHAEACEAE	Pink
Millingtonia hortensis Linn.	Indian cork tree	Peep	BIGNONIACEAE	White
Rhinacanthus nasutus (Linn.) Kurz.	-	Thong pun chang	ACANTHACEAE	White
Punica granatum Linn.	Pomegranate	Thap thim	PUNICACEAE	Red

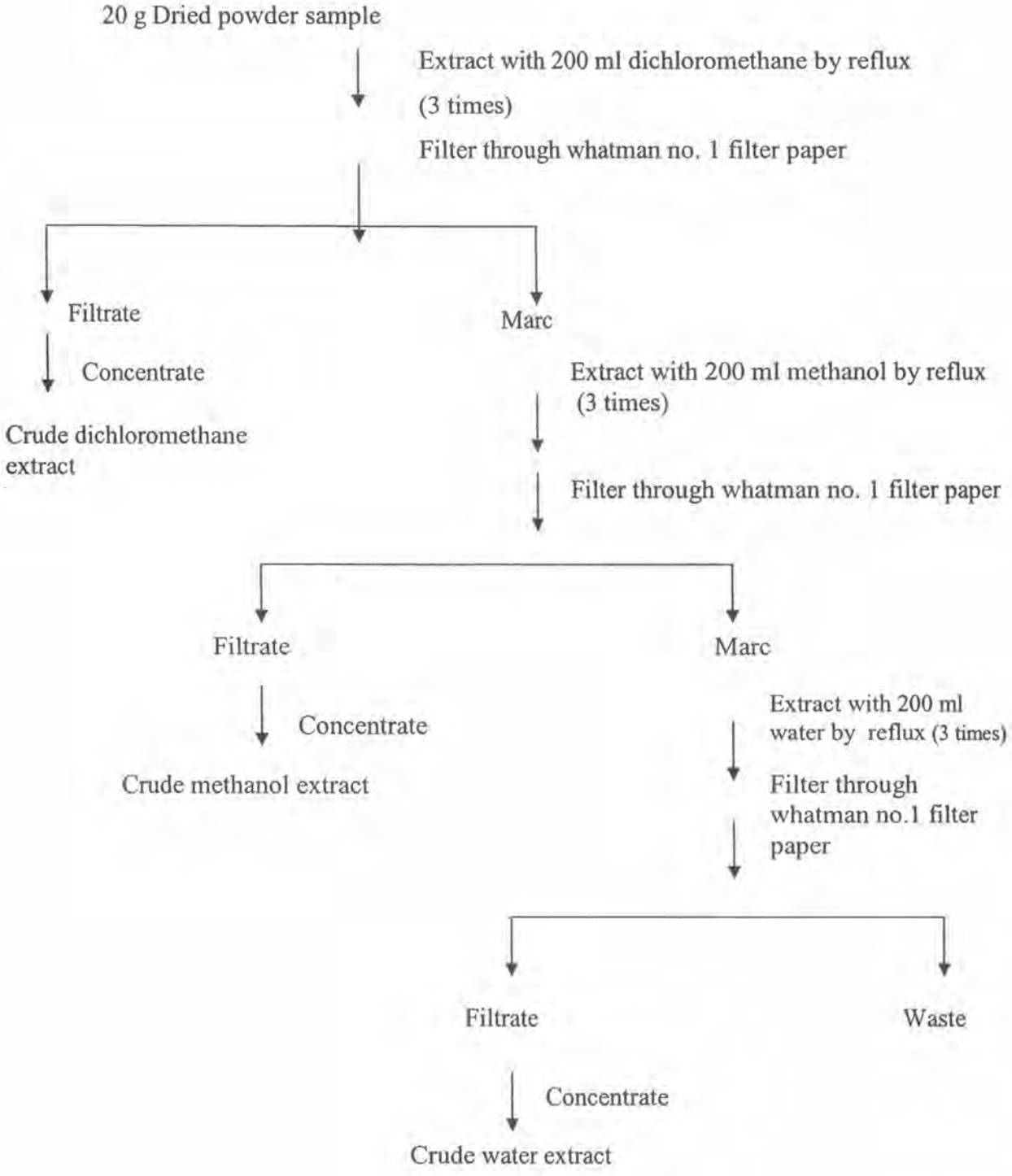

Figure 19. The steps of flower extraction

3.2 Experimental Design

Overall information required to elucidate the study of flower extracts was shown in Figure 20. Biological activities including cytotoxicity, antioxidative activity, mutagenicity and antimutagenicity of each flower extract were evaluated. The cytotoxicity study was performed using brine shrimp assay (Meyer *et al.*, 1982). In addition, antioxidative activity including DPPH and FRAP assay were performed. The mutagenic and antimutagenic effects were evaluated using the Ames test (Yahagi *et al.*, 1975) and the somatic mutation and recombination test (SMART) (Graf *et al.*, 1984). Furthermore, total phenolic content was performed using Folin-Ciocalteu colorimetric method.

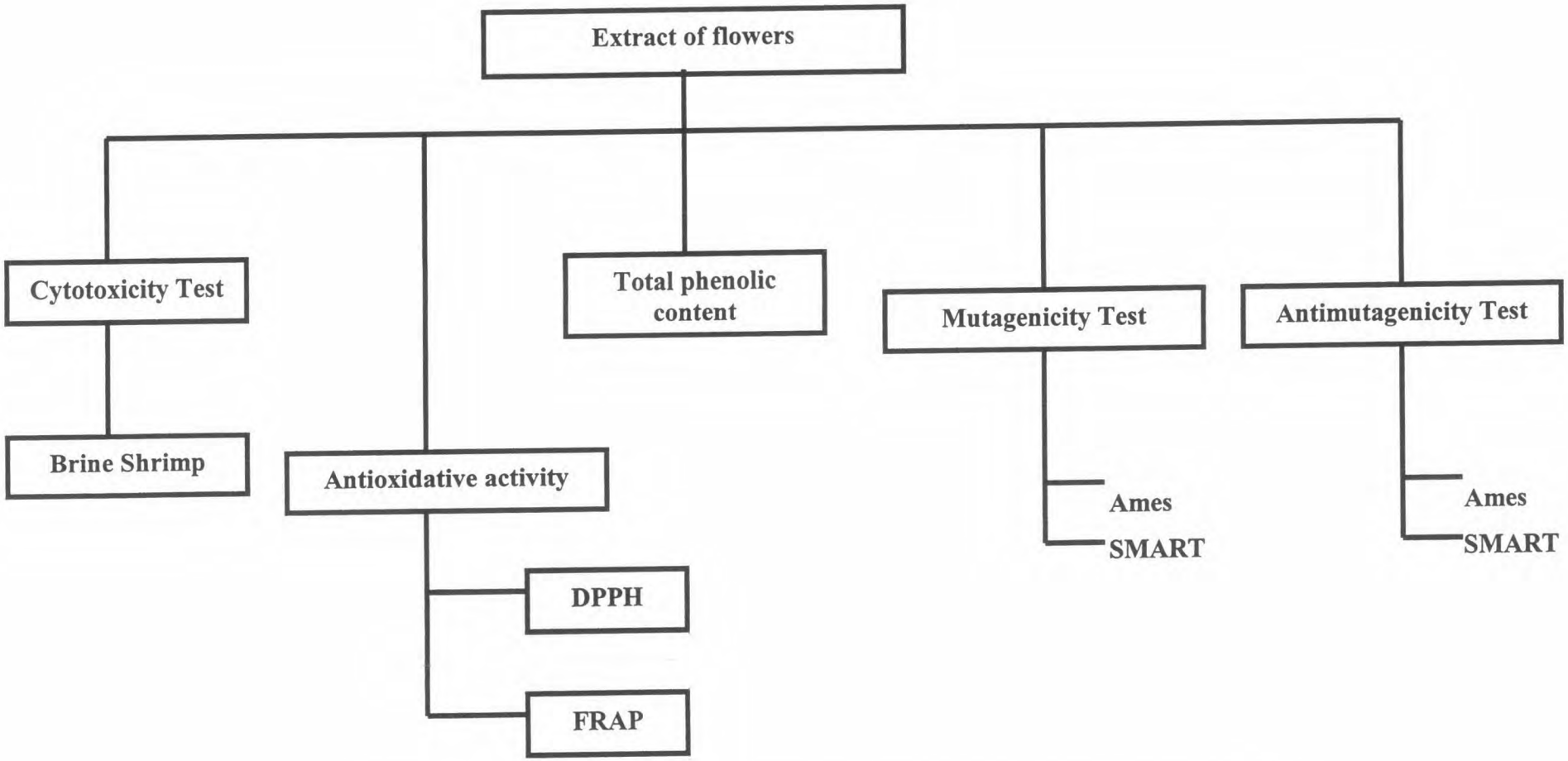

Figure 20. Overall investigations to elucidate the biological activities of selected flowers

3.3 Brine Shrimp Assay

3.3.1 Chemical

Brine shrimp eggs and artificial sea water (Q-SEA) were purchased from fish market. Component in artificial sea water was shown in Appendix A.

3.3.2 Sample Preparation

Samples were prepared by dissolving 50 mg of dry extract in 1 ml of methanol (methanol extract and water extract of flowers) or dichloromethane (dichloromethane extract of flowers) (solution A). Solution B was prepared by diluting 0.1 ml of A to 1 ml with methanol (methanol extract and water extract of flowers) or dichloromethane (dichloromethane extract of flowers). Solution C was prepared by diluting 0.1 ml of B to 1 ml with methanol (methanol extract and water extract of flowers) or dichloromethane (dichloromethane extract of flowers). 100 µl of solution A, B and C (1000, 100 and 10 µg/ml, respectively) were transferred to 1.25 cm (1/2 in) discs of filter paper. The discs were dried in air, placed in 2 dram vials. Control disc were prepared using methanol or dichloromethane. Five replicates were prepared for each dose level.

3.3.3 Hatching the Shrimp

Brine shrimp eggs were hatched in shallow rectangular dish filled with artificial sea water which was prepared with a commercial salt mixture and distilled water. A plastic divider with several 2 mm holes was clamped in the dish to make two unequal compartments. The eggs were sprinkled into the larger compartment which was darkened, while the smaller compartment was illuminated. After 48 hours the phototropic nauplii (brine shrimp larvae) were collected by pipette from the lighted side, having been separated by divider from their shells (Meyer *et al.*, 1982)

3.3.4 Bioassay

Ten shrimp were transferred to each sample vial using disposable pipette, and artificial sea water was added to make 5 ml. Paper disc from 3.3.2 are placed in vials containing brine shrimp of five replicates. The nauplii can be counted macroscopically in the stem of the pipette against a lighted back ground. Hatched brine shrimp nauplii can survive for up to 48 h without food because they still feed on their yolk-sac (Pelka *et al.*, 2000). The vials were maintained under illumination. Survivors were counted after 6 and 24 hours and the percent deaths at each dose and control were determined. The 24 hours counts were more useful. Percent deaths were

calculated as: % deaths = percentage of survival in the control- percentage of survival in the treatment (Carballo *et al.*, 2002)

The LC_{50} values of the extracts were obtained by a plot of percentage of the shrimp nauplii killed against the concentrations of the extracts and the best-fit line was obtained from the data by means of regression analysis (Krishnaraju *et al.*, 2005) (Appendix A).

3.4 Antioxidant Assay

3.4.1 Chemicals

TPTZ (2, 4, 6-tripyridyl-s-triazine), ferric chloride hexahydrate, and ferrous sulfate heptahydrate were purchased from Sigma Chemical (St. Louis, MO, USA). Diethylether, Potassium hydroxide, and Sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). Propionic acid, DPPH (2, 2′ - diphenyl-1-picrylhydrazyl), Gallic acid and Folin-Ciocalteu reagent were purchased from Fluka Chemika (Buchs, Switzerland). Trolox was purchased from Aldrich Chemical (Milwaukee, WI, Germany). Glacial acetic acid was obtained from J.T. Baker (Phillibsburg, USA). Sodium carbonate anhydrous was purchased from Riedel-De Haen AG (Seelze, West Germany). Hydrochloric acid was purchased from Lab Scan Ltd. (Dublin, Ireland). Other chemicals were of laboratory grade.

3.4.2 Sample Preparation

Dichloromethane extract

0.05 g of dried dichloromethane extracts were dissolved with dimethy sulfoxide (1 ml) (solution 1). Pipette 100 µl from solution 1 and adjust volume to 500 µl with dimethy sulfoxide (solution 2). Next, pipette 100 µl from solution 2 and adjust volume to 500 µl with dimethy sulfoxide for used in below experiment.

Methanol and water extract

0.05 g of dried methanol and water extracts were dissolved with 80% methanol (1 ml). (solution 1). Next procedure for dilution was described as the same dichloromethane extract.

3.4.3 2,2′-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

The antioxidant activity of each flower extracts on DPPH was estimated using the procedure described by Fukumoto and Mazza (2000) with slight modifications.

An aliquot of 22 µl (0.044 mg/ml) (in triplicate) of each extract or standard Trolox was transferred into a 96-well flat-bottom micro plate (Bibby Sterilin Ltd, UK). The solution of 150 µM DPPH in 80% methanol (200 µl) was added to each micro plate well. The plate was then covered and left to stand in the darkness at room temperature. After 30 min, the absorbance of the solution was read in a micro plate reader (Sunrise, Tecan Co., Austria) at 520 nm. The standard curve was constructed by using several concentrations of Trolox (0.08 - 1.28 mM in 80% methanol) (Appendix B). The antioxidant activity of the extracts was determined using the standard curve expressed as mg of Trolox Equivalent Antioxidant Capacity (TEAC)/g dry weight of sample.

3.4.4 Ferric Reducing Antioxidant Power (FRAP) Assay

The antioxidant activity was measured by its ability to reduce the Fe^{3+}/ferricyanide complex by forming ferrous products. Ferrous (Fe^{2+}) can be monitored by measuring the formation of Perl's Prussian blue at 600 nm. Increased absorbance at 600 nm indicates a stronger reducing power. Each (22 µl) (0.044 mg/ml) extract or standard or blank reagent was added to each well in a 96-well micro titer plate in triplicate. FRAP reagent (150 µl), freshly prepared (see Appendix B) and warmed at 37°C according to the procedure described by Griffin and Bhagooli (2004), was added to each well. The mixture was left at room temperature for 8 min. The absorbance was read at 600 nm using a micro plate reader. The change in absorbance after 8 min from the initial blank reading was compared to that of a standard that was run simultaneously. Aqueous solutions of known standard Fe^{2+} ($FeSO_4.7H_2O$) concentrations (62.5, 125, 250, 500, 1000 µM) were used for calibration (Appendix B). The FRAP values of the extracts were determined using this standard curve, expressed as mg of ferrous iron (Fe (II))/g dry weight of sample. Data were presented as means ± SD of triplicate wells.

3.4.5 Determination of Total Phenolic Contents

The total phenolic content of each extract from each sample was determined according to method described by Swain and Hillis (1959), Naczk and Shahidi (1989) and Amarowicz *et al.* (2004) with slight modification by using a micro plate reader. Briefly, 10 µl (0.02 mg/ml) of each extract was transferred into a 96-well flat-bottom micro plate containing 160 µl of distilled water. After mixing the contents, 10 µl of Folin-Ciocalteu reagent and 20 µl of saturated sodium carbonate solution were added.

The micro plate was vortex and absorbance of blue colored mixtures was recorded after 30 min at 750 nm with micro plate reader (Sunrise, Tecan Co., Austria). The amount of total phenolic content was calculated as a Gallic Acid Equivalent (GAE) from the calibration curve of gallic acid standard solutions (concentration range between 25 and 800 mg/l), and expressed as mg gallic acid equivalent/g dry weight of sample (Appendix B). All measurements were done in triplicate. Data were presented as means ± SD.

3.5 Ames Test

3.5.1 Chemicals

1-Aminopyrene (Aldrich, St. Louis, U.S.A.) was used to interact with nitrite in acid solution to produce a standard direct mutagen of the Ames test. Acetonitrile was purchased from Mallinckrodt Inc. Ampicillin was purchased from Vesco pharmaceutical Ltd. (Bangkok, Thailand). D-Biotin, ammonium sulfamate were obtained from Sigma Chemical Company (St. Louis, Missouri, U.S.A.). D (+)-Glucose monohydrate, crystal violet indicator and sodium ammonium hydrogen phosphate tetrahydrate GR were bought from Fluka AG (Buch, Switzerland). E. Merck (Darmstadt, Germany) supplied L-histidine monohydrochloride, sodium chloride, hydrochloric acid, magnesium sulfate heptahydrate, citric acid monohydrate GR, potassium chloride, di-sodium hydrogenphosphate, dimethylsulfoxide (DMSO) and Bacto agar. Oxoid nutrient broth No. 2 was supplied by Oxoid Ltd., (Basingstoke, Hants, England). Sodium dihydrogen phosphate was obtained from May & Baker Ltd., (Degenham, England). Sodium nitrite and di-potassium hydrogen phosphate anhydrous were purchased from BDH Chemicals Ltd. (Poole, England).

3.5.2 Nutrient Agar

3.5.2.1 Preparation of Minimal Agar Plate

Minimal agar containing 1.5 % Bacto agar was autoclaved and then it was mixed with 2 % sterile glucose and Vogel-Bonner medium E (Appendix C). About 30 ml of molten top agar was poured on to a sterile Petri dish. It was left until solidified and stored at 37°C in the incubator.

3.5.2.2 Preparation of Top Agar

Top agar containing 0.6 % Bacto agar and 0.5 % sodium chloride was autoclaved and was stored at room temperature. Before use, the agar was melted and

10 % of a sterile solution of 0.5 % mM histidine and biotin was added to the molten top agar and then it was maintained at 45°C in a water bath.

3.5.3 Bacterial Tester Strain

Salmonella typhimurium tester strains used in this study were histidine dependent strains (His^-) TA98 and TA100 which were capable of detecting frameshift mutation and base-pair substitution, respectively. Both strains were kindly provided by Dr.Kaew Kansadalampai, Mahidol university, Thailand. The tester strains were manipulated as suggested by Maron and Ames (1983). Overnight culture of each tester strain incubated from frozen stock culture in oxoid nutrient broth No. 2 at 37°C was used for mutagenesis assay (Appendix C). Cultures were kept refrigerated until use.

3.5.4 Mutagenicity of flower extracts

3.5.4.1 Mutagenicity of flower extracts without nitrite treatment

An aliquot (10, 50, 100 and 200 µl) of each flower extract was added to the tube containing appropriate amount of distilled water or dimethyl sulfoxide to obtain the final volume of 200 µl. 0.2N HCl containing sufficient acid to acidify the reaction mixture to pH 3.0-3.5 was added until the pH of mixture was stable and added 250 µl of distilled water or dimethyl sulfoxide to the reaction. The final volume was 1000 µl. The reaction tube was shaken at 37 °C for 4 h. The reaction was stopped by allowing the mixture to stand for 1 min in an ice bath. Then, 250 µl of distilled water or dimethyl sulfoxide was added to the reaction mixture and then the reaction tube was immersed in an ice bath for 10 min. Then, the mixture was determined for its mutagenicity by Ames test using *Salmonella typhimurium* TA 98 and TA 100. It was shown in Figure 21.

The pre-incubation method as suggested by Yahagi *et al.* (1975) was used throughout this study. The mixture (100 µl) was mixed with 500 µl of 0.5 M phosphate buffer (pH 7.4), 100 µl of each tester strain and it was incubated at 37 °C in shaking water bath for 20 min. After incubation, 2 ml of top agar containing 0.5 mM L-histidine and 0.5 mM D-biotin (45°C) was added, mixed well and poured onto a minimal glucose agar plate. The plate was rotated to achieve uniform colony distribution and incubated at 37 °C in darkness for 48 h. Number of His^+ revertant colonies were counted. A compound was classified as a mutagen if the results satisfied two criteria (1) a dose dependent increase in the number of revertants was observed and (2) the number of revertants was equal to or greater than two times of

the negative control (Mortelmans and Zeiger, 2000). The tube containing DMSO or water (negative control) was used to determine spontaneous reversion.

3.5.4.2 Mutagenicity of flower extracts with nitrite treatment

An aliquot (10, 50, 100 and 200 µl) of flower extract from experiment was added to the tube containing appropriate amount of distilled water or dimethyl sulfoxide to obtain the final volume of 200 µl. 0.2N HCl containing sufficient acid to acidify the reaction mixture to pH 3.0-3.5 was added until the pH of mixture was stable. Adding 250 µl of 2M sodium nitrite started the reaction. The final volume was 1000 µl. The reaction tube was shaken at 37 °C for 4 h. The reaction was stopped by allowing the mixture to stand for 1 min in an ice bath. Then, 250 µl of 2M ammonium sulfamate was added to the reaction mixture to decompose the residual nitrite and then the reaction tube was immersed in an ice bath for 10 min. Then, the mixture was determined for its mutagenicity by Ames test using on *Salmonella typhimurium* TA 98 and TA 100. A volume of 100 µl of nitrite treated flower extracts were tested for their mutagenicity as described above (3.5.4.1). It was shown in Figure 21.

3.5.5 Standard Direct Mutagens

1-Aminopyrene (AP) treated with nitrite in acid solution was used as a positive mutagen since it gave direct-acting mutagenicity in the condition similar to that occurred during stomach digestion (Kangsadalampai, Kusamran, and Butryee. 1995; Kangsadalampai, Butryee, and Manoonphol, 1996). Briefly, 10 µl (testing on *Salmonella typhimurium* TA 98 of the Ames test) or 20 µl (testing on *Salmonella typhimurium* TA 100 of the Ames test) of 1-aminopyrine (0.075 mg/ml) in a tube fitted with a plastic stopper was mixed with 730-740 µl of 0.2 N hydrochloric acid (sufficient to acidify the reaction mixture to pH 3-3.5) then 250 µl of 2 M sodium nitrite was added to the reaction mixture. The reaction tube was shaken at 37 °C for 4 h and the reaction was stopped by placing the tube in an ice bath for 1 min. 2 M ammonium sulfamate (250 µl) was added to the tube, mixed well, and the whole was allowed to stand for 10 min in an ice bath before it was used as the positive standard control. Then, the mixture was determined for its mutagenicity by Ames test using on *Salmonella typhimurium* TA 98 and TA 100. A volume of 100 µl of nitrite treated 1-aminopyrene was tested for their mutagenicity using pre-incubation method (Yahagi *et al.* 1975). It was used as a positive control (Kangsadalampai *et al.*, 1996).

3.5.6 Effect of the Flower Extracts on the Standard Mutagen

For antimutagenicity testing, 40 µl (testing on *Salmonella typhimurium* TA 98 of the Ames test) or 80 µl (testing on *Salmonella typhimurium* TA 100 of the Ames test) of 1-aminopyrine (0.075 mg/ml) in a tube fitted with a plastic stopper was mixed with 730-740 µl of 0.2 N hydrochloric acid (sufficient to acidify the reaction mixture to pH 3-3.5) then 250 µl of 2 M sodium nitrite was added to the reaction mixture. The reaction tube was shaken at 37 °C for 4 h and the reaction was stopped by placing the tube in an ice bath for 1 min. 2 M ammonium sulfamate (250 µl) was added to the tube, mixed well, and the whole was allowed to stand for 10 min in an ice bath. A volume of 25 µl of nitrite treated 1-aminopyrene was tested for antimutagenicity. Flower extracts solution (25, 50 and 75 µl) were transferred into a sterile plastic stopped tube containing 25 µl of mutagen (nitrite-treated 1-aminopyrene). Adjust volume to 100 µl with dimethyl sulfoxide (for dichloromethane and methanol extract of flowers) or adjust volume to 100 µl with water (for water extract of flowers). The mixture (100 µl) was mixed with 500 µl of 0.5 M phosphate buffer (pH 7.4), 100 µl of each tester strain and it was incubated at 37 °C in shaking water bath for 20 min. After incubation, 2 ml of top agar containing 0.5 mM L-histidine and 0.5 mM D-biotin (45°C) was added, mixed well and poured onto a minimal glucose agar plate. The plate was incubated at 37 °C for 48 h and revertant colonies were then counted (Figure 22).

3.5.7 Data evaluation

The results were reported as mean and standard deviation of histidine (His^+) revertants per plate. A compound was classified as a mutagen if the results satisfied two criteria (1) a dose dependent increase in the number of revertants was observed and (2) the number of revertants was equal to or greater than two times of the negative control (Mortelmans and Zeiger, 2000). The percentage of modification (either increase or decrease on mutagenicity of standard direct mutagens) was calculated as following:

$$\% \text{ Modification} = \frac{(A - B)}{(A - C)} \times 100$$

Where A is number of histidine revertants per plate induced by nitrite treated 1-aminopyrine, B is number of histidine revertants per plate induced by nitrite treated

1-aminopyrine in the presence of each sample and C is number of spontaneous histidine revertants per plate.

In terms of antimutagenic activity, it is classified as suggested by Calomme *et al.*(1996) as following:

more than 60%	strongly inhibition;
41-60%	moderately inhibition;
21-40%	weakly inhibition;
0-20%	negligible inhibition.

In terms of increase mutagenicity, it is classified as suggested by Calomme *et al.*(1996) as following:

0 to -20%	negligible enhancement;
-40 to -21%	weak enhancement;
-60 to -41%	moderately enhancement;
more than -60%	strongly enhancement.

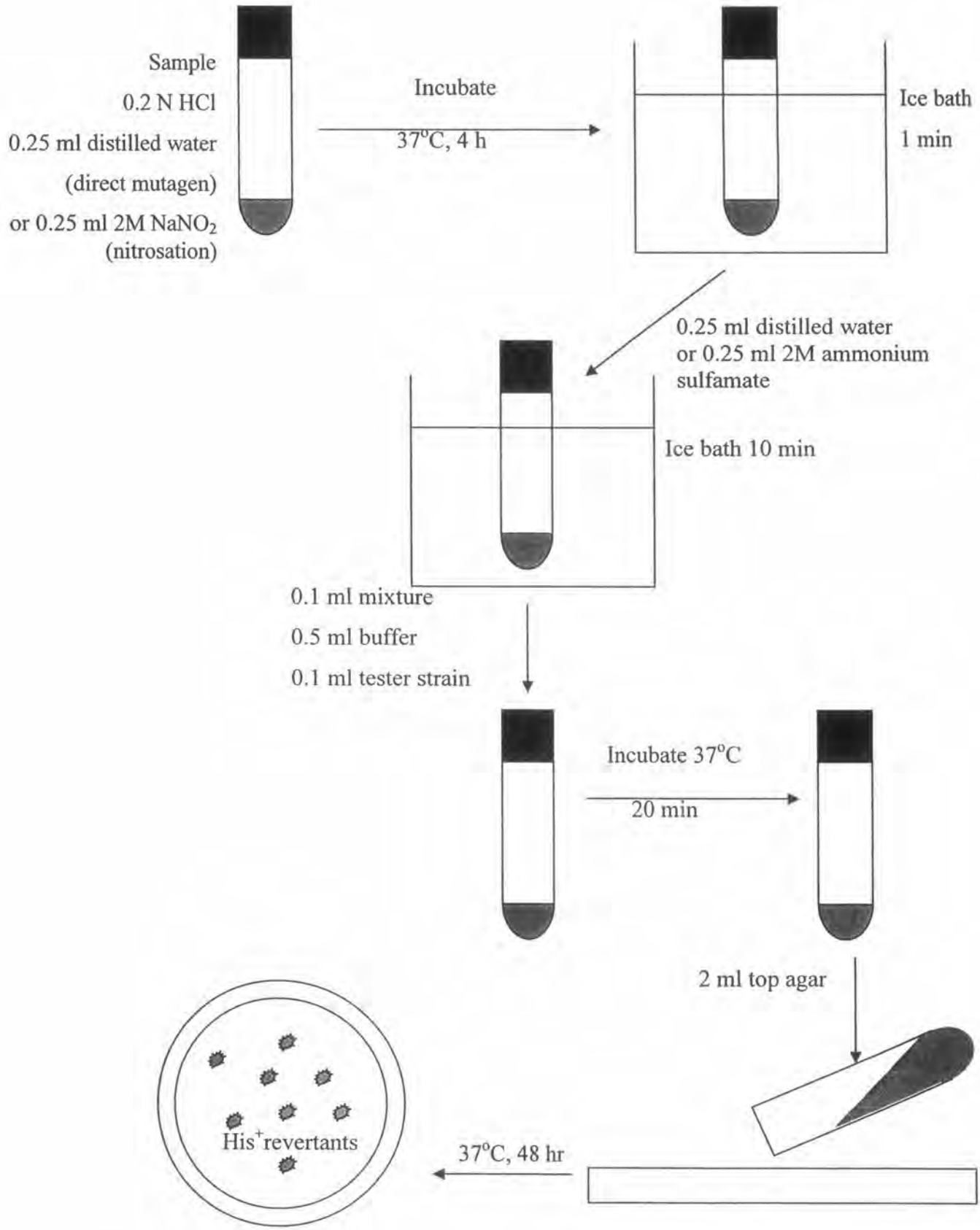

Figure 21. Steps to determine the mutagenicity of samples using the Ames mutagnicity test (pre-incubation modification) in the absence of S-9 mix (Yahagi *et al.*,1975).

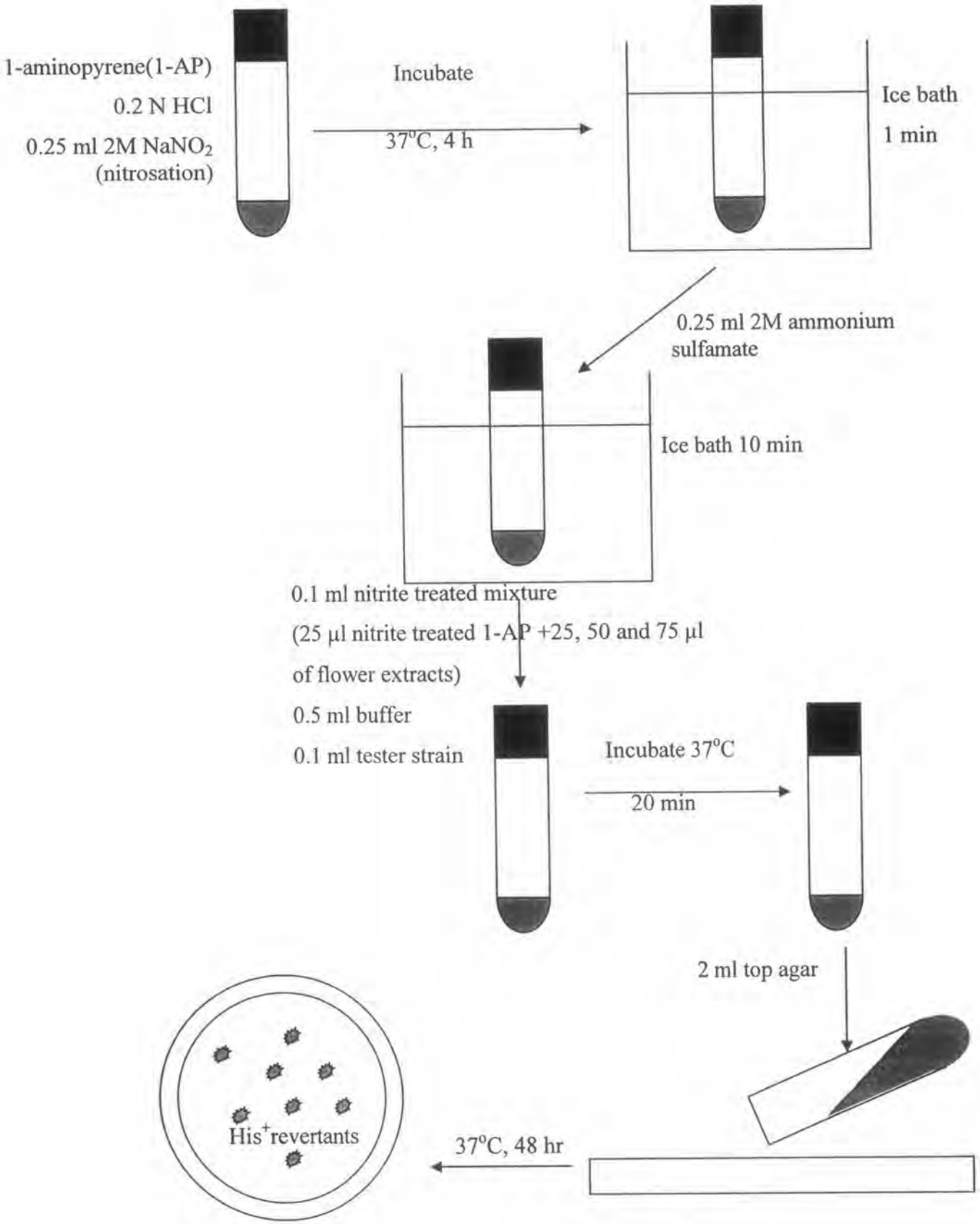

Figure 22. Steps to determine the antimutagenicity of samples using the Ames mutagnicity test (pre-incubation modification) in the absence of S-9 mix (Yahagi *et al.*,1975).

3.6 Somatic Mutation and Recombination Test (SMART)

3.6.1 Chemicals

Urethane was purchased from Sigma chemical (St. Louis, MO, USA). Glycerol was bought from Farmitalia Carlo Erla (Milan, Italy). Gum arabic powder was purchased from BDH Chemical Ltd. (Poole, England). Chloral hydrate was supplied by Srichand United Dispendary Co. Ltd. (Thailand). Other chemicals were of laboratory grade.

3.6.2 *Drosophila melanogaster* Strains

Two *Drosophila melanogaster* strains were used. Virgin females of *ORR/ORR; flr3/In(3LR)TM3, ri pp sep l(3)89Aa bx34e e Bds* were crossed with males of *mwh/mwh*. Prof. U. Graf (University of Zurich, Switzerland) kindly provided both strains. The stocks were kept on the regular medium composed of corn flour 125 g, sugar 100 g, baker's yeast 50 g, agar 14 g, propionic acid 5 ml and water 1000 ml (Robert,1986).The cultures of the flies as well as the treated larvae were maintained at a constant temperature 16±1 °C and 25±1 °C respectively.

3.6.3 Regular *Drosophila* Medium

Yeast-glucose-agar Drosophila medium was the formula of Robert (1986). It composed of corn flour (3.75 g), sugar (3.00 g), agar (0.45 g), and yeast (1.50g). The ingredients were mixed and boiled in a 50 ml Erlenmeyer flask containing 30 ml deionized water until it became sticky. Propionic acid was added (0.15 ml) to the stock of fly culture, mating and collecting larvae.

3.6.4 Sample Medium

Types of media used in this study were shown in table 5. 0.4 gram of each dichloromethane, methanol and water extract of red hibiscus, Mexican creeper, ixora, white frangipani, malay apple, kra chiew, sacred lotus, Indian cork tree, thong pun chang, and pomegranate were mixed in the standard medium using mortal and pestle. All media were studied for their mutagenicity and antimutagenicity. Negative control and positive control contained distilled water and 2 ml of 20 mM urethane, respectively.

Table 5 Composition of media

Component of medium	Standard medium or Negative control	Positive control medium	Experimental medium (Co-administration)	Sample medium
Corn flour	0.25 g	0.25 g	0.25 g	0.25 g
Sugar	0.20 g	0.20 g	0.20 g	0.20 g
Baker's yeast	0.10 g	0.10 g	0.10 g	0.10 g
Agar	0.03 g	0.03 g	0.03 g	0.03 g
Water	2 ml	-	-	2 ml
Urethane	-	2 ml (20mM)	2 ml (20mM)	-
Flower extracts	-	-	0.4 g	0.4 g

3.6.5 Survival and Mutagenicity Study

Each sample was studied for its mutagenicity as described by Graf *et al.* (1984). Virgin females of *ORR; flr*^{3} were mated with *mwh* males on the standard medium. Six days after mating, 100 of 3-day old larvae (72 h) were collected, washed with water, and transferred (with the help of a fine artist's brush) to experimental medium that contained varied concentration of each flower extracts, negative control and medium containing urethane (positive control). They were incubated at 25±1°C until pupation. After metamorphosis, the surviving flies were collected and stored in 70% ethanol. Survival rates of adult flies from larva fed on all experimental media were collected to determine the toxicity of each sample. Only the media that provide more than 50% survival of flies was determined for its mutagenicity.

The insect bearing the marker trans-heterozygous (*mwh+/+flr*^{3}) indicated with round wings were mounted on a microscope slide. Wings were separated from the body with a fine paintbrush, lined up on a clean slide. A droplet of Faure's solution (30 g gum arabic, 20 ml glycerol, 50 g chloral hydrate and 50 ml deionized water) as suggested by Graf *et al.* (1984) was dropped on the slide and a cover slip was put on. The round wings of surviving flies (both the dorsal and ventral surface), at least 40 wings, were analyzed under a compound microscope at 400x magnification for the presence of clones of cells showing malformed wing hairs. The position of the spots was noted according to the sector of the wing (Figure 23). Different types of spots namely, single spots showing either the multiple wing hairs *(mwh)* or the flare

(*flr*^{3}) phenotype, and twin spots showing adjacent *mwh* and *flr*^{3} areas were recorded separately. The size of each spot was determined by counting the number of wing cells (hairs) exhibiting the *mwh* or the *flr*^{3} phenotype. The spots were counted as two spots if they were separated by three or more wide-type cell rows. Multiple wing hairs (*mwh*) were classified when a wing cell contained three or more hairs instead of one hair per cell as in wide-type. Flare wing hairs exhibited a quite variable expression, ranging from pointed, shortened and thickened hairs to amorphic, sometimes balloon-like extrusions of melanolic chitinous material.

The wing spots data were evaluated using the statistical procedure described by Frei and Würgler (1988). Frequencies of induced wing spots of both the treated groups and the negative control (deionized water treated group) were compared. The resulting wing spots were classified accordingly into the following: (1) small single spots of 1 or 2 cells in size, (2) large single spots of 3 or more cells, and (3) twin spots. The estimation of spot frequencies and confidence limits of the estimated mutation frequency were performed with significance level of $\alpha = \beta = 0.05$. A multiple decision procedure was used to decide whether a result was positive, weakly positive, inconclusive or negative according to Frei and Würgler (1988). Statistical consideration and calculation step by step are shown in Appendix D.

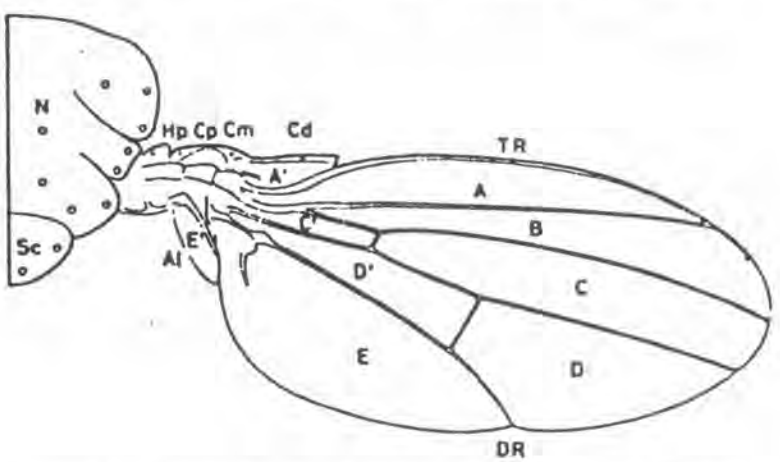

Figure 23. Normal half mesothorax showing the regions A-E of the wing surface scored for spots according to Graf *et al.* (1984).

3.6.6 Antimutagenicity Study

All samples that provided more than 50% survival of adult flies and did not express their genotoxicity were evaluated in co-administration (Figure 24) The experimental medium was prepared by adding each highest concentration of dichloromethane, methanol and water extracts in the standard medium.

3.6.6.1 Co-administration Study

Virgin *ORR;flr*^{3} females and *mwh* males were mated on the standard medium. Six days after mating, 100 of 3-day old larvae (72 h) were collected, washed with water and transferred (with the help of a fine artist's brush) to each experimental medium containing 20 mM urethane, negative control and positive control medium. They were incubated at 25±1°C until pupation. The surviving adult flies were collected after pupation and were progressed as of mutagenicity study.

The percentage of inhibition was also calculated as following $100(a-b)/a$ where a is the frequency of spots induced by urethane alone and b the frequency of spots induced by urethane in the presence of sample (Abraham, 1994). It is proposed that percentage of inhibition between 0-20 represented a negligible effect while expression of percent inhibition between 20-40, 40-60 and more than 60 are the evidence of weak, moderate and strong antimutagenicity, respectively.

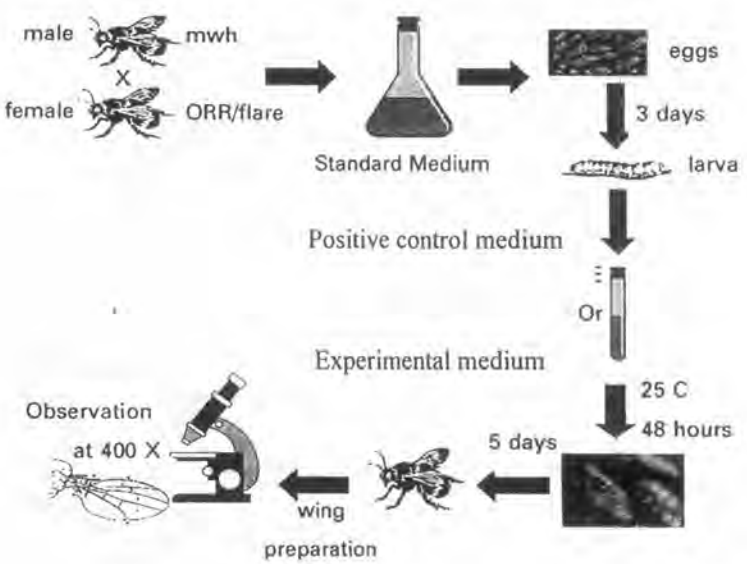

Figure 24. Co-administration study of sample on mutagenicity of urethane induced wing spots of *Drosophila melanogaster* (By courtesy of Assoc. Prof. Kaew Kangsadalampai).