CHAPTER IV
MATERIALS AND METHODS

4.1 Sample Preparation

Cockroach berry or Ma Khuea Pro in Thai and yellow berried nightshade or Ma Khuea Lueng in Thai have the same scientific name as *Solanum aculeatissimum* Jacq. While aubergine or Ma Khuea Muang Glom in Thai is *Solanum melongena* L. Their pictures are shown in Figure 4.1. Each eggplant (1-3 kg) was purchased randomly from three retail stores in Salaya community market (Nakhonpathom, Thailand) and pooled as a composite sample. Each sample was washed with tap water several times to remove adhering contaminants. The edible portion of each eggplant was separated, washed again with deionized water and drained on a stainless sieve until dry.

Cockroach berry or Ma Khuea Pro

Yellow Berried Nightshade or Ma Khuea Lueng

Aubergine or Ma Khuea Muang Glom

Figure 4.1 Different eggplants in this study

The sample was divided into 3 portions. The first one was cut into small pieces, homogenized in an electrical blender, lyophilized to be powder and served as a raw sample. The second one was cut into small pieces (2 mm thickness), steamed for 2 min, homogenized in an electrical blender, lyophilized to be powder and labeled as steamed sample. In order to reduce the uptake of oil by fried sample as suggested by Debnath *et al*. (2003) the last portion was cut into small pieces (2 mm thickness), steamed for 2 min, lyophilized, fried at 120-140˚C for 10 s in palm oil, and labeled as fried sample. The practice assigned to fried sample preparation also inhibited polyphenol oxidases (Yamaguchi *et al*., 2003) that were activated after vegetables

were cut and exposed to oxygen. These enzymes can oxidize polyphenolic compounds and the phenomenon is the cause of loss of their radical-scavenging activity (Takamura *et al*., 2002). The samples were kept in vacuum bags and stored in desiccators at room temperature for further studies. The experiments were done twice (in *separate* day) as shown in Figure 4.2.

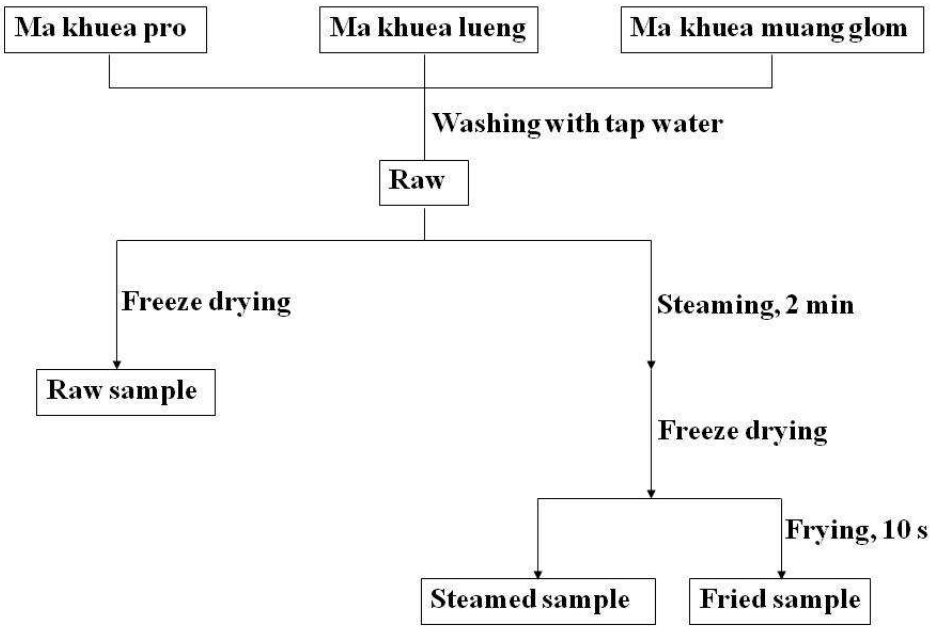

Figure 4.2 The preparation of eggplant samples for further determinations.

4.2 Experimental Design

Overall of the experiment design to evaluate the benefit to consumer of the eggplants selected for the present study was shown in Figure 4.3. Samples were prepared twice and separately determined as described below.

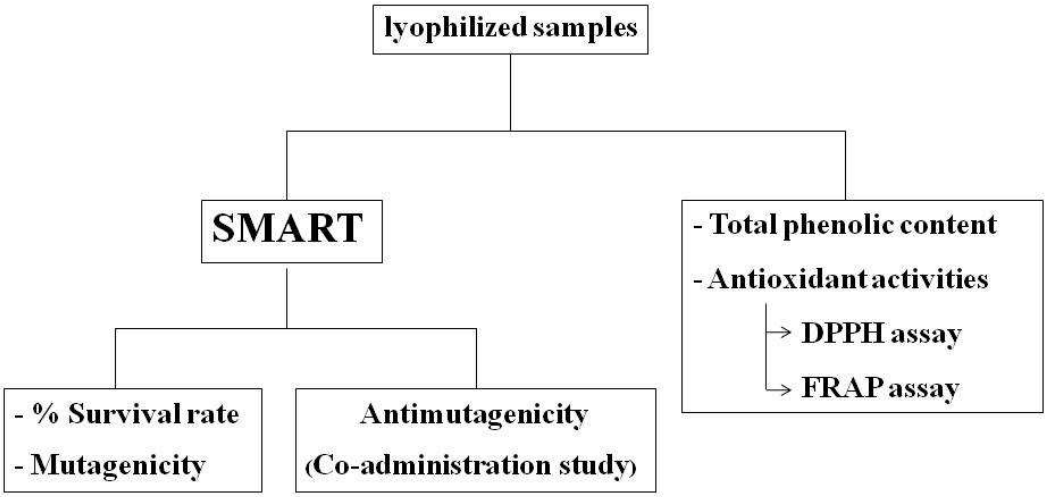

Figure 4.3 Experimental designs to evaluate antioxidant activities, mutagenicity and antimutagenicity of each sample

4.3 Antioxidant Activity Assay

4.3.1 Chemicals

Fluka Chemika (Buchs, Switzerland) supplied 2, 2- diphenyl-1-picrylhydrazl (DPPH), gallic acid and Folin-Ciocalteu reagent. Sodium acetate trihydrate was purchased from Merck (Darmstadt, Germany). Sodium carbonate anhydrous was obtained from Riedel-De Haen AG (Seelze, West Germany). Trolox was furnished by Aldrich Chemical (Milwaukee, WI, Germany). 2, 4, 6-tripyridyl-s-triazine (TPTZ), ferric chloride hexahydrate, and ferrous sulfate heptahydrate were obtained from Sigma Chemical (St. Louis, Missouri, USA). Other chemicals were of laboratory grade.

4.3.2 Sample Extraction

Each sample (0.5 g) was stirred with 80% methanol (50 ml) at room temperature for 2 h. The solution was filtered through Whatman filter paper No. 1. Each extract was assayed for its total phenolic content and antioxidant activities.

4.3.3 Determination of Total Phenolic Content

The total phenolic content of methanol extract from each sample was determined according to the method described by Amarowicz *et al*. (2004) with slight modification as suggested by Kruawan and Kangsadalampai (2006). Briefly, 10 µl of each extract was transferred into 96-well microplate containing 160 µl of distilled water. After mixing, 10 µl of Folin-Ciocalteu reagent and 20 µl of a saturated sodium carbonate solution were added. The solution was mixed well and the absorbance was measured at 750 nm after 30 min incubation using ELISA plate reader model Sunrise (Tecan Co., Austria). The total phenolic content of each sample was calculated from a calibration curve of gallic acid solutions (ranging from 25 to 800 mg/l), and was expressed as mg of gallic acid equivalents (GAE) per g dry weight. All measurements were done in triplicate.

4.3.4 DPPH Assay for Free Radical Scavenging Activity

The antioxidant activity of the extract from sample on stable radical 2, 2-diphenyl-1-picrylhydrazl (DPPH) was estimated using the procedure described by Fukumoto and Mazza (2000) with some modifications. An aliquot of 22 µl of the extract or blank reagent (80% methanol) or standard Trolox (0.04-1.28 mM in 80% methanol) was added to 200 µl of DPPH in 80% methanol (150 µM) in a 96 well flat bottom microplate (Bibby Sterilin Ltd, UK). After incubation at 37^{o}C for 30 min, the absorbance of the solution was read using a ELISA plate reader (Sunrise, Tecan Co., Austria) using a 520 nm filter. The antioxidant activity of the extract was determined using the standard curve expressed as mg of Trolox Equivalent Antioxidant Capacity (TEAC) per g dry weight. The radical scavenging activity was calculated as a percentage of DPPH scavenging activity using the equation (Amarowicz *et al*., 2004):

DPPH scavenging activity (%) = 100 x [1-(A_E/A_D)]

Where A_E is the absorbance of the solution containing DPPH and the extract, and A_D is the absorbance of only the DPPH solution.

4.3.5 Ferric Reducing Antioxidant Power (FRAP) Assay

The antioxidant activity was measured by its ability to reduce the Fe^{3+}-TPTZ complex by forming Fe^{2+}-TPTZ and could be monitored by measuring the

formation of Perl's Prussian blue at 600 nm. The working FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2, 4, 6-tripyridyl-s-triazine (TPTZ) solution and 20 mM $FeCl_3.6H_2O$ in a 10:1:1 ratio prior to use and warmed to 37^oC in water bath according to the procedure described by Griffin and Bhagooli (2004). Aliquots of known ferrous sulfate concentrations (62.5, 125, 250, 500, 1000 μM) were used for calibration. An aliquot (20 μl) of each extract or of standard (ferrous sulfate) or of the reagent blank (80% methanol) was added to each well of a 96-well microplate and run in triplicate. The 150 μl of FRAP reagent was added to each well. The change in absorbance from the initial blank was recorded after 8 min of incubation using an ELISA plate reader (Sunrise, Tecan Co., Austria) using a 600 nm filter. The FRAP values of the extracts were determined using this standard curve, expressed as mg of ferric reduced per g dry weight.

4.4 Mutagenicity Assay

4.4.1 Chemicals

Urethane was purchased from Sigma Chemical (St. Louis, Missouri, USA). Chloral hydrate was supplied by Srichand United Dispendary Co., Ltd (Thailand). Glycerol was bought from Farmitalia Carlo Erla (Milan, Italy). Gum Arabic powder was purchased from BDH Chemical Ltd. (Poole, England). Propionic acid was purchased from Fluka Chemika (Buchs, Switzerland). Other chemicals were of laboratory grade.

4.4.2 Drosophila Media

4.4.2.1 Standard Medium Yeast-glucose-agar *Drosophila* media was prepared as suggested by Robert (1986). It composed of corn flour (3.75 g), sugar (3.00 g), agar (0.45 g) and yeast (1.50 g). The ingredients were mixed and boiled in 50 ml Erlenmeyer flask containing 30 ml deionized water until it became sticky. Propionic acid was added (0.15 ml) to the medium as a preservative. This medium was used for maintaining the stock of fly culture, mating and collecting larvae. The preparation step by step of *Drosophila* medium is shown in Appendix A.

4.4.2.2 Experimental Medium Each lyophilized sample (0.15 g, 0.29 g or 0.58 g) was mixed well with 0.58 g fly medium containing all components but water in a beaker to obtain an experimental medium with the 12.5, 25 or 50 percent sample addition, respectively. Then 0.58 g of the mixture was transferred to a test tube and 2 ml deionized water was added. The mixture was boiled until it became sticky. This experimental medium was used for mutagenicity determination and also as a sample control in antimutagenicity determination. The standard medium described above was used as a negative control while the standard medium containing 20 mM urethane was used as a positive control.

4.4.3 Somatic Mutation and Recombination Test

The test was carried out according to the method described by Graf *et al*. (1984). Two *Drosophila melanogaster* strains kindly provided by Professor U. Graf (University of Zurich, Switzerland) were used. Twenty virgin females of *ORR; flr*^{3} strain were mated with twenty-five males of *mwh/mwh* strain to produce trans-heterozygous larvae of improved high bioactivation cross (IHB). Six days after mating, 100 of 3-day old larvae (72 h) were collected, washed with water (used a fine artist's brush) and transferred to experimental medium, positive control medium (standard medium containing urethane 20 mM or 1.78 mg/ml) or negative control medium (standard medium). All larvae were incubated at 25±1^{o}C until pupation. On days 10-12 after egg lying, the insect bearing the marker trans-heterozygous (*mwh*+/+ *flr*^{3}) indicated with round wings were collected and stored in 70% ethanol as suggested by Graf and van Schaik (1992).

The round wings of surviving flies were washed with distilled water and 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) was dropped on the slide and a cover slip was put on. The 40 round wings of surviving flies (both the dorsal and ventral surface) of each treatment 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 4.4). Different type of spots namely, single spots found either on the multiple

wing hairs (*mwh*) or the flare (*flr*^{3}) phenotype, and twin spots found on 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 wild-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 shown in wild-type. Flare wing hairs (*flr*^{3}) exhibited a quite variable expression, ranging from pointed, shortened and thickened hairs to amorphic, sometimes ballon-like extrusions of melanolic chitinous material.

The wing spots data was 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 as indicated in Figure 4.5: (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 spots frequencies and confidence limits due to mutation 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. Statistical consideration and step by step calculation are shown in Appendix B.

4.4.4 Antimutagenicity assay The highest concentration of each sample providing more than 50% survival was selected to determine for its antimutagenicity. The experimental design is shown in Figure 4.6. A portion of lyophilized sample (0.58 g) was mixed well with 0.58 g fly medium containing all components but water in a beaker; then 0.58 g of the mixture was transferred to a test tube and 2 ml 20 mM urethane solution was added. The mixture was boiled until it became sticky. This experimental medium containing 20 mM urethane was used for antimutagenicity. The standard medium described above was used as a negative control while the standard medium containing 20 mM urethane was used as a positive control.

Twenty virgin females of *ORR; flr*^{3} strain were mated with twenty-five males of *mwh/mwh* strain on the standard medium. Six days after mating, 100 of 3-day old larvae (72 h) were collected, washed with water (used a fine artist's brush) and transferred to experimental medium containing urethane (20 mM), negative control or

positive control medium (see 4.3.2.2). They were incubated at 25±1°C until pupation. The surviving adult files were collected after pupation and were proceed as of mutagenicity study. The percentage of modification (inhibition or induction) was calculated (Abraham, 1994) as following:

Percentage of modification (inhibition or induction) = (*a*-*b*)/*a* x 100

When *a* is the frequency of spots induced by urethane alone and *b* the frequency of spots induced by urethane in the presence of sample. 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 were the evidences of weak, moderate and strong antimutagenicity, respectively.

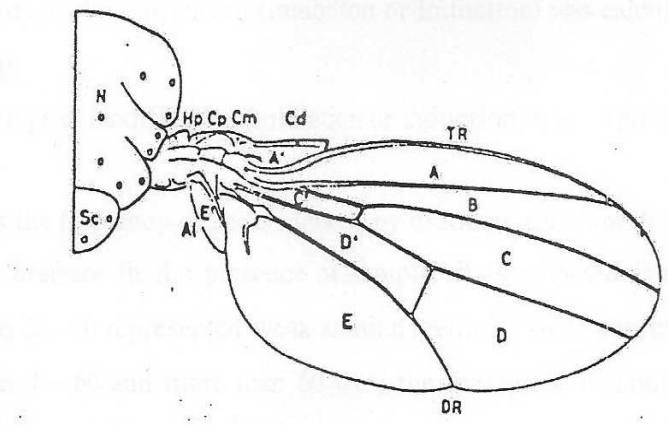

Figure 4.4 Normal half mesothorax showing the regions A-E of the wing surface scored for spots according to Graf *et al*. (1984).

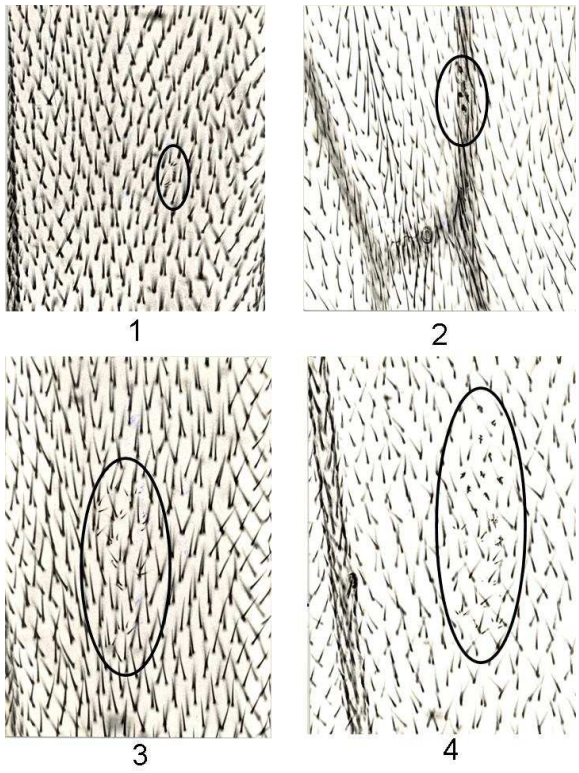

Figure 4.5 Difference types of wing hair mutation, 1) small single spots of *mwh* on wing, 2) large single spots of flare on wing, 3) large single spots of *mwh* on wing, 4) twin spots (By courtesy of Assoc. Prof. Kaew Kangsadalampai).

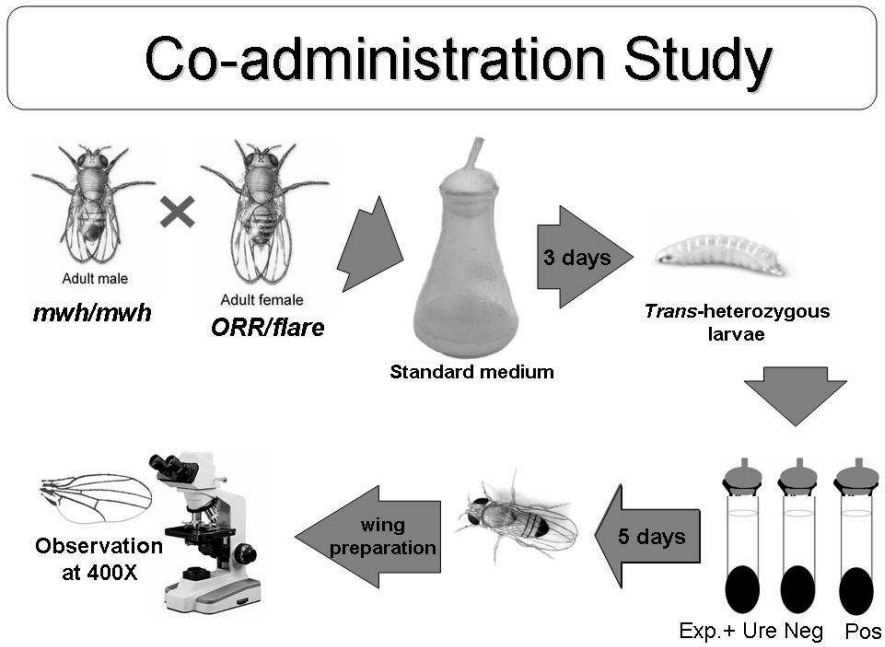

Figure 4.6 Co-administration study of each selected eggplant on mutagenicity of urethane induced wing spots of *Drosophila melanogaster*