CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals for SMART

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

3.2 *Drosophila* Tester Strain and Cross

Two stains of *Drosophila melanogaster* were used. Virgin females of Oregon wing flare strain (*ORR/ORR; flr^3/TM3, ser*) were mated with males multiple wing hairs strain (*mwh/mwh*). Both strains were kindly provided by Professor U. Graft (University of Zurich, Switzerland).

3.3 Experimental Design and Sample Preparation

The experimental design for overall investigation to elucidate the effect of mushroom samples is shown in figure 10. Each mushroom extract was studied for its effect on survival rate of adult flies. The suitable concentration that gave more than 50% survival flies was obtained by substituting distilled water (the solvent of the medium) with 2 ml of each mushroom extracts for bringing up larvae to be adult flies. Then the mutagenicity test of each sample was performed using in order to ensure that sample was not mutagenic. Finally, the antimutagenicity against standard mutagen, urethane, of each sample was also conducted.

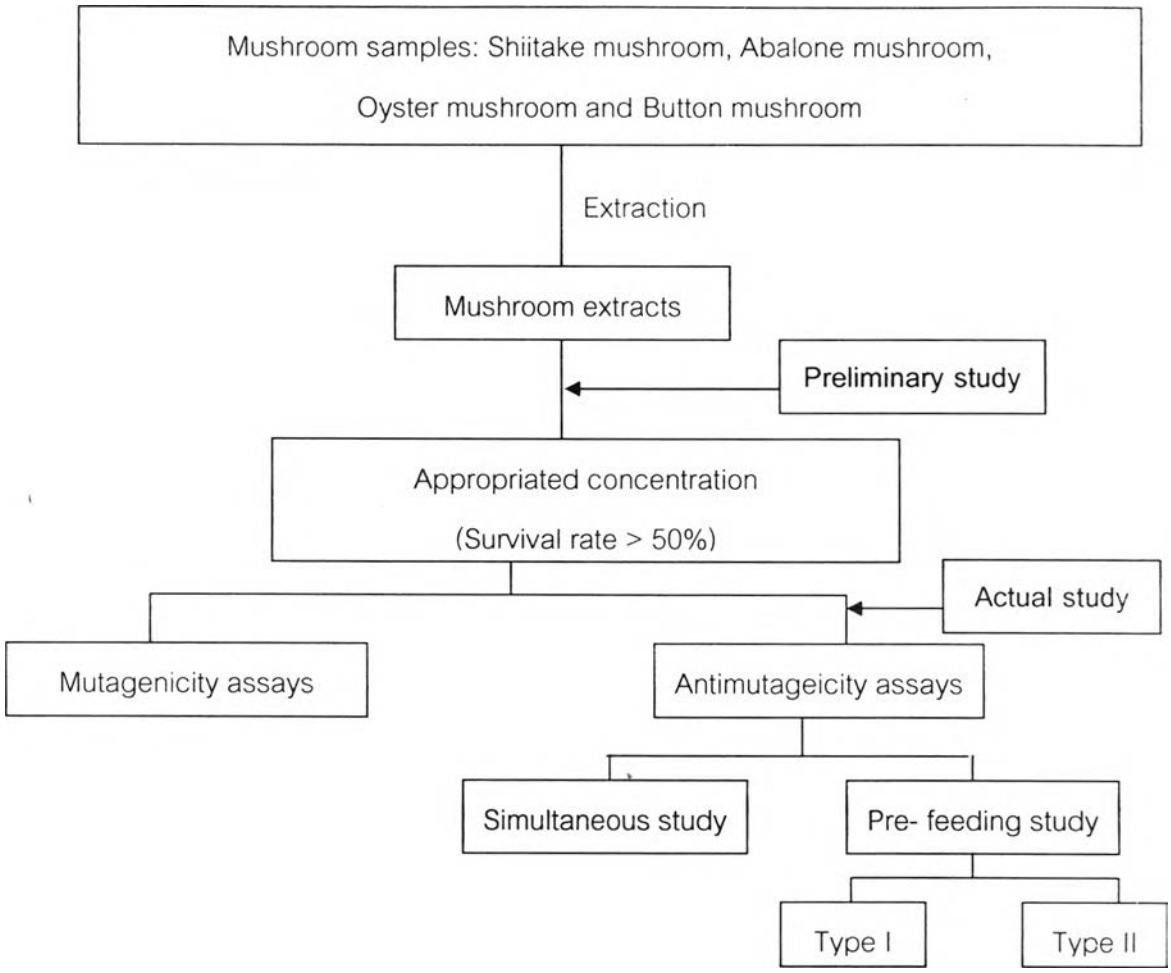

Figure 10 Overall investigations to elucidate the effect of mushroom samples

3.3.1 Sample preparation

Samples of *Lentinus edodes*, *Agaricus bisporus*, *Pleurotus ostreatus*, *Pleurotus abalonus* were purchased from a local market in Bangkok. The fruiting bodies (cap and stalk) of all fresh mushrooms were washed; air- dried and blended in a home- use electrical blender. Each sample was divided into three portions.

First portion was used as a fresh sample and other portions were processed with either boiling or pickling. Fresh mushroom extract was prepared by homogenizing 100 g untreated mushroom with 100 ml distilled water using the Ultra-Turrax T25 homogenizer (IKA Labortechnik, Janke and Kunkel GMBH and Co. KG, Staufeni, Germany). The homogenate was filtered through with gauzes and filter paper (Whatman No.1) to obtain filtrate.

Second portion was blanched mushroom extract. This extracts prepared by adding 100 g untreated mushroom to 100 ml of boiling distilled water and left it at room temperature. After cooling, the samples was homogenized by an Ultra- Turrax T25 homogenizer and then filtered through with gauzes and filter paper (Whatman No.1).

The last portion was fermented mushroom extract. This portion was prepared from untreated mushroom 100 g boiling with 100 ml of 4% w/v NaCl solution for 5 min, after cooling, keep it in plastic container overnight, and homogenized by an Ultra-Turrax T25 homogenizer. The homogenate was filtered through with gauzes and filter paper (Whatman No.1). Each filtrate was used immediately for media preparation. The steps for sample preparations were shown in Figure 11.

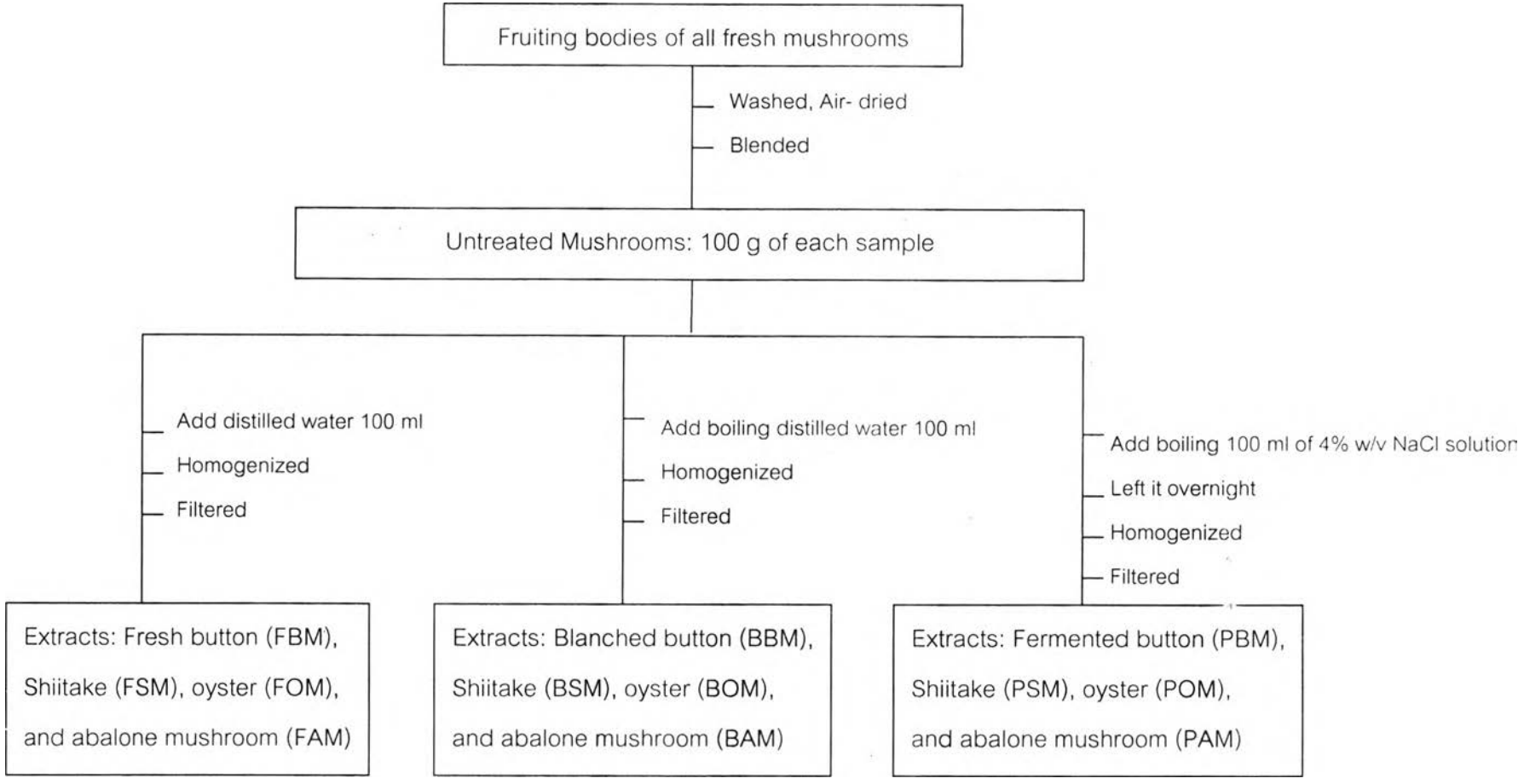

Figure 11 Steps for sample preparations

3.3.2 *Drosophila* Stock and Experimental Medium

Drosophila Stock

Two stains of *Drosophila melanogaster* were used. Virgin females of Oregon wing flare hair strain (*ORR/ORR; flr*^{3}*/TM3, ser*) were mated with males multiple wing hair strain (*mwh/mwh*). Those laboratory stocks were kept on standard medium (Robert, 1986). The culture of the flies as well as the treated larvae was maintained at a constant temperature 25±1°C.

Medium for *Drosophila* Stock

Standard medium Standard medium was composed of 0.25 g corn flour, 0.2 g sugar, 0.10 g baker's yeast, 0.03 g agar, 0.1 ml propionic acid and 2 ml water. The preparation step by step of *Drosophila* medium (standard medium) is shown in Appendix.

And type of media for ***experiment*** shown in Table 12.

Table 12 Type of media for *experiment*

Composition of medium	Diluents	Type of media for experiment
0.25 g corn flour 0.2 g sugar 0.10 g baker's yeast 0.1 ml propionic acid	2 ml water	Standard medium (negative control medium)
	2 ml of 20 mM urethane solution	Positive control medium
	2 ml of the mushroom extracts	Mutagenic sample medium
	1 ml distilled water and 1 ml of mushroom extracts	Sample medium
	1 ml of 40 mM urethane solution and 1 ml of mushroom extracts	Experimental medum

Standard medium This medium was used for negative control.

Positive control medium This medium was used for positive control medium.

Mutagenic sample medium This medium was used for evaluating the mutagenicity assay of sample extracts.

Sample medium This kind of medium was used for mutagenicity assay.

Experimental medium This medium was used for evaluating the antimutagenesis assay of sample extracts.

3.3.3 Preliminary Studies

3.3.3.1 Survival of adult flies

The mushroom extract was studied for its effect on survival rate of adult flies. The suitable concentration that gave more than 50% survival was obtained by substituting distilled water (the solvent of the medium) with 2 ml of the mushroom extract for bringing up larvae to be adult flies. Then the mutagenicity test of each sample was performed in order to ensure that the sample was not mutagenic.

3.3.3.2. Mutagenicity Assay

The mutagenicity assay of each extract was carried out as described by Graf *et al.* (1984). Virgin females of Oregon wing flare (*ORR*; *flr*^{3}*/TM3 ser)* were allowed to mate with multiple wing hair males *(mwh/mwh)* to produce trans-heterozygous (*mwh+/+flr*^{3}) larvae of improved high bioactivation cross (IHB) fed in standard medium. The 3-day old larvae (72 h) were collected, washed with water and transferred (with the help of a fine artist's brush) to the mutagenic sample medium, positive control medium and negative control medium. Larvae were maintained on the culture medium at 25$\pm$1°C for 48 h until pupation. After metamorphosis, the survived flies were collected and stored in 70% ethanol from the tubes between days 10-12 after egg laying (Figure 13). Only the insect bearing the marker trans-heterozygous (*mwh+/+flr*^{3}) indicated with round wings were collected as suggested by Graf and Van (1992). Their wings were mounted on microscope slides and were examined for mutant spots. Numbers of survived flies were evaluated to obtain the suitable concentration for antimutagenicity study.

3.3.4 Actual Studies

After the suitable concentration that provided more than 50% survival was obtained, antimutagenicicty of each sample extract was evaluated. The experiments were designed to be simultaneous feeding and pre-feeding studies.

Antimutagenicity Assay Experiments were conducted in two assays. One was a simultaneous feeding study and the other was a pre-feeding study. In the simultaneous feeding study (Figure 14), the virgin *ORR; flr*^{3} /TM3 females and *mwh*/mwh males were mated in the standard medium. Six days after mating, the 3-day-old larvae were transferred in equal batches to experimental medium, sample medium, positive control medium and negative control medium.

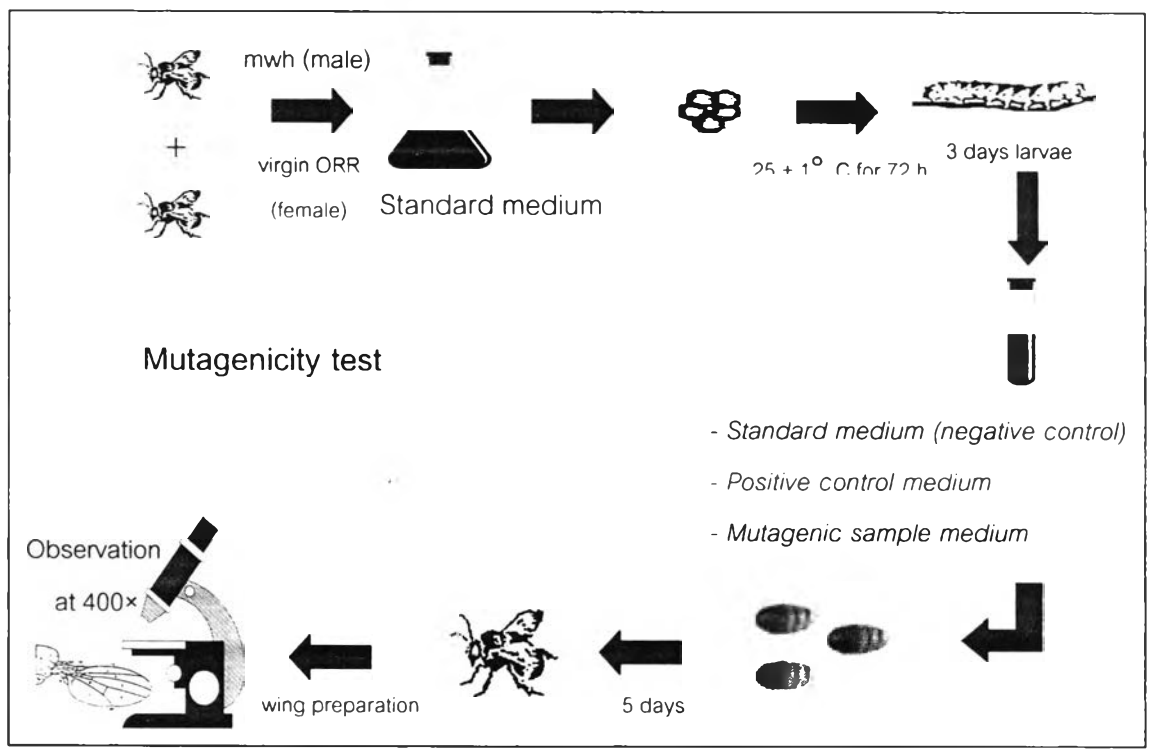

Figure 13 Mutagenicity test of the mushroom extracts.

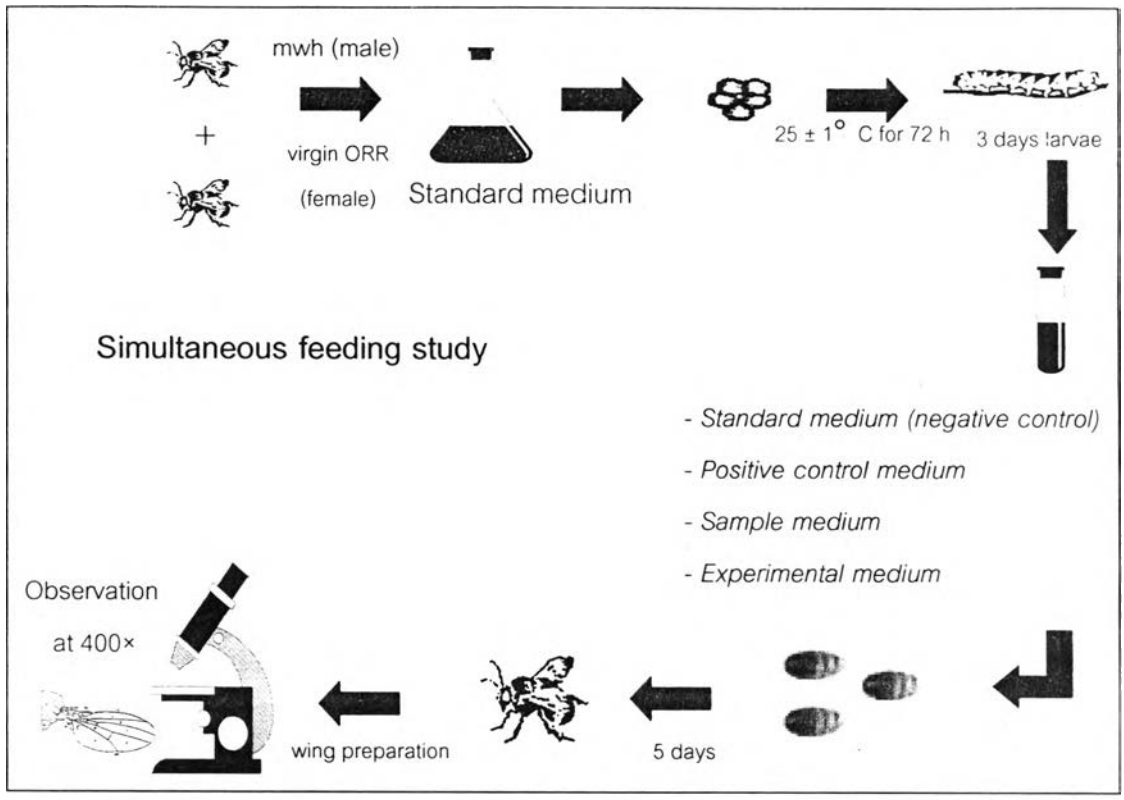

Figure 14 Simultaneous feeding study of the mushroom extracts.

In pre-feeding study (Figure 15), there were 2 types of experiments. The virgin *ORR;flr*^{3} */TM3* females and *mwh/mwh* males were allowed to mate on the sample medium to obtain 3-day-old larvae. Then, the larvae were transferred to a positive control medium, which was designed as a type I experiment and the second medium was an experimental medium [2 ml of distilled water was substituted with 1ml of mushroom extract and 1 ml of urethane (40 mM)] it was designed as type II experiment. Type I experiment was designed to observe whether the sample had a continuous effect in modulating biotransformation of urethane while type II would reveal whether the sample needed to be administered all duration of larval development to be pupa. Larvae were maintained on the medium until pupation (48 h). The survived adult flies were collected on days 7-10 after pupation. Only the insect bearing the marker trans-heterozygous (*mwh+/+flr*^{3}) indicated with round wings was stored in 70% ethanol. Subsequently, the wings were removed, mounted and scored under a compound microscope for recording of the wing spot as described by Graf *et al.* (1989).

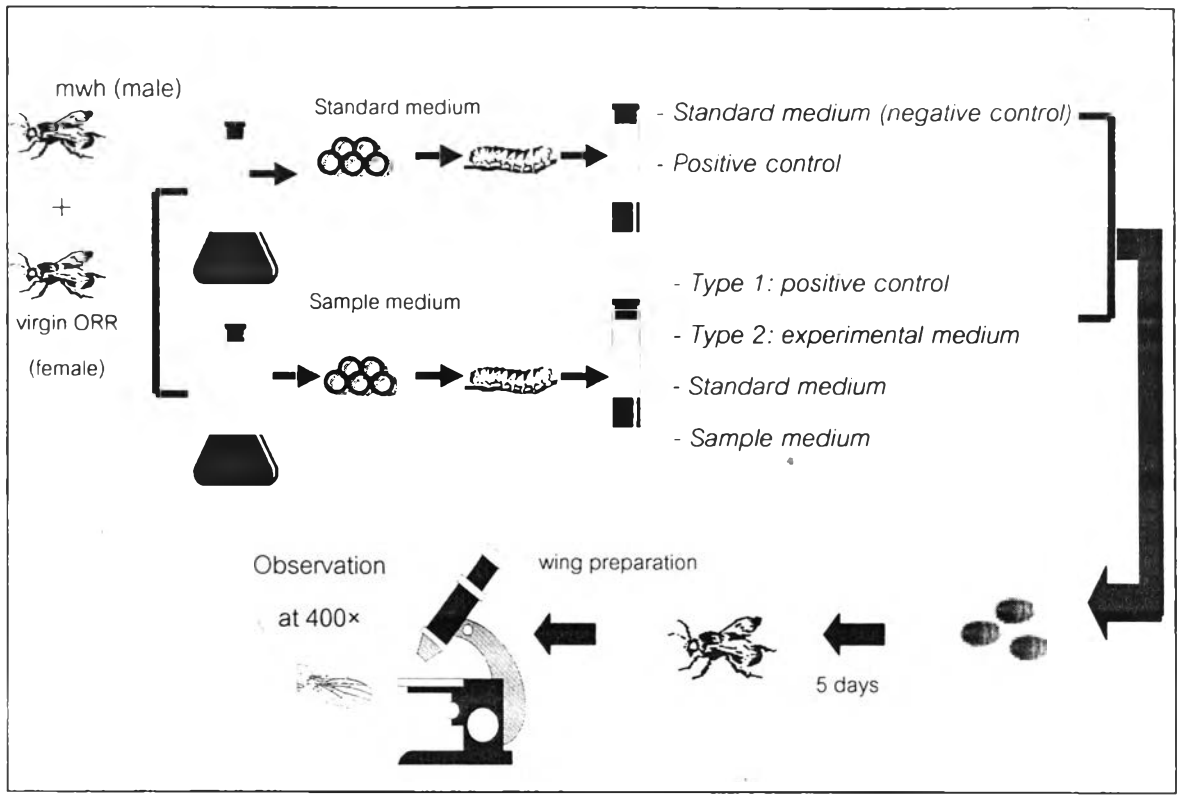

Figure 15 Pre- feeding study of the mushroom sample on mutagenicity of urethane in *Drosophila melanogaster*.

3.4 Wing Preparation

After metamorphosis, the survival flies were collected from the tubes days 10-12 after egg laying and stored in 70% ethanol. Only the insect bearing the maker trans-heterozygous (*mwh+/+flr*^{3}) indicated by round wings were collected as suggested by Graf and Schaik (1992) and their wings were mounted on microscope slides (described below); thus they were examined for the mutant spots. The flies in 70% ethanol were washed with distilled water. Wing were separated from the body with a sharp blade then were lined up on a clean slide by a fine paintbrush. A droplet of Faure's solution was composed of 30 g gum arabic, 20 ml glycerol, 50 g chloral hydrate and 50 ml distilled wate as suggested by Graf *et al.* (1984) was dropped on the slide and a coverslip was put on. The round wings of surviving flies (both the dorsal and ventral surfaces), at least 40 wings, were analyzed under the 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 16). Different types of the spots namely, single spots showing either 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 mutant 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 wings cell containing three or more hairs instead of one hair per cell as in wild type (Figure 17). Flare wing hair exhibited a quite variable expression, ranking from pointed, shortened, and thickened hairs to amorphic, sometimes balloon-like extrusion of melanolic chitinous material.

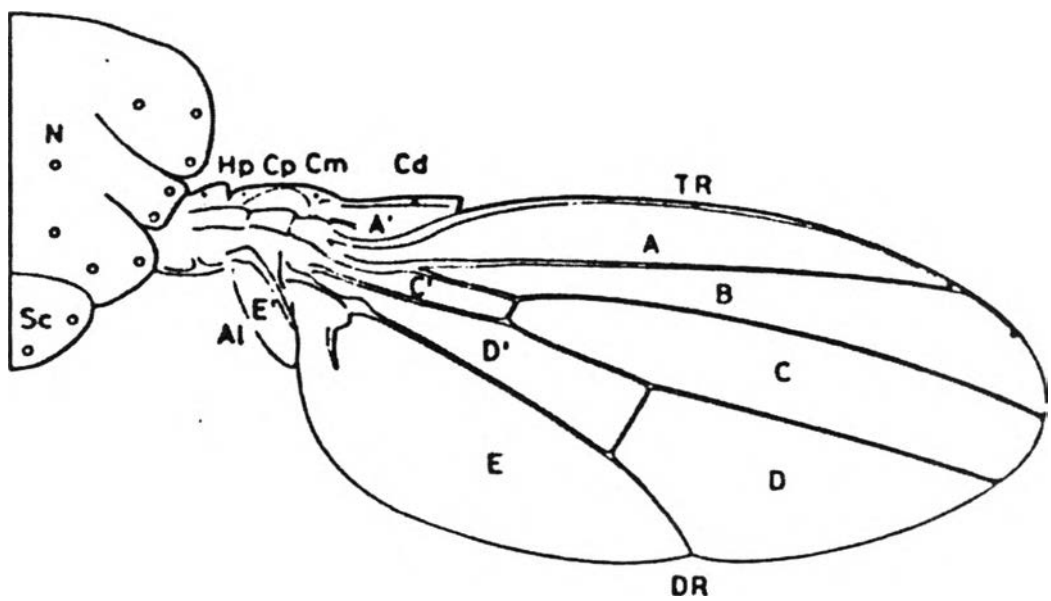

Figure 16 Normal half mesothorax showing the regions A- E of the wing surface scored for spots according to Graf *et al.* (1984)

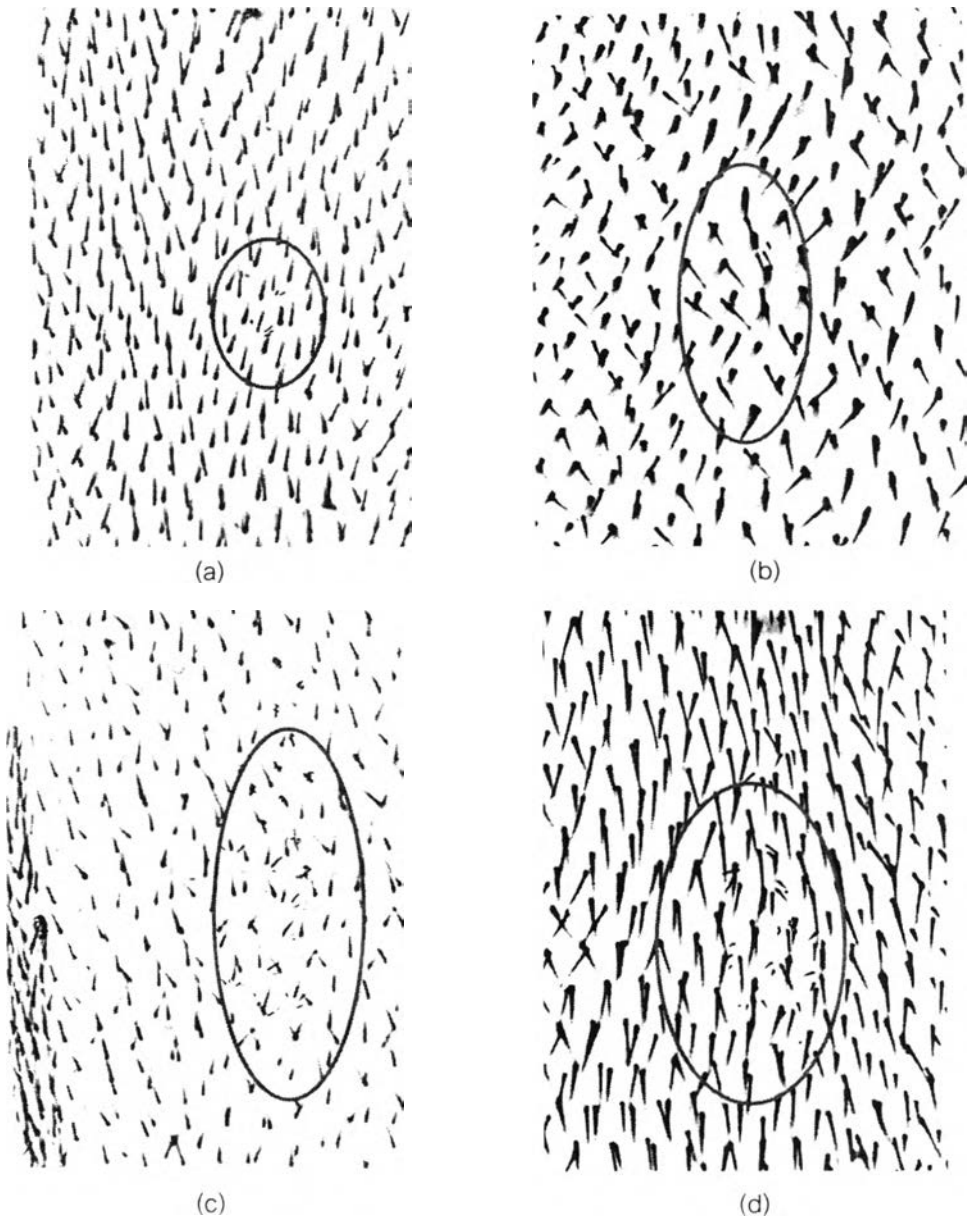

Figure 17 Trichromes on the wing blade, a) small single spots, b) flare on wing, c) twin spots, d) large single spots

3.5 Data Evaluation and Statistical Analysis

3.5.1 Mutagenicity test

The wing spot data were evaluated using the statistical procedure described by Frei and Wurgler (1988). Concisely, induction frequencies of wing spots of crude mushroom extracts treated groups were compared with those of negative control group (distilled water). The spots were grouped accordingly to the following 3 types: (1) small single spots of 1 or 2 cells, (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 Wurgler (1988). Statistical considerations and calculation step by step are shown in Appendix.

3.5.2 Mutagenicity modulation

The mutagenic modulation by each sample was estimated as suggested by Negishi *et al.* (1994). Mutagenicity index of urethane obtained from simultaneous and pre-feeding studies were compared with that from a urethane group. Mutagenicity index of urethane (M.I.) was calculated as the following.

$$\text{M.I.} = \frac{\text{A number of spot per wing induced with URE administrated with sample}}{\text{A number of spot per wing induced with URE only}}$$

Generally, M.I. of URE is 1. The M.I. will decrease if the sample contains any antimutagen or will increase in the presence of mutagenicity potentiator.

M.I. = > 0.80 - 1.00: no effect

M.I. = 0.60 - 0.80: weak antimutagenicity

M.I. = 0.40 - 0.60: moderate antimutagenicity

M.I. = < 0.40: strong or potent antimutagenicity

M.I. = 1.00 – 1.20: no effect

M.I. = 1.20- 1.40 weak mutagenicity potentiator

M.I. = 1.40- 1.60 moderate mutagenicity potentiator

M.I. = > 1.60 strong mutagenicity potentiator