CHAPTER III

MATERIALS AND METHODS

3.1 The plant materials

3.1.1 Plant powder preparation (Cherdshewasart *et al.*, 2004[a])

The tuberous roots of the wild *P. mirifica* were collected from 28 provinces of Thailand, including Chiang Rai, Chiang Mai, Mae Hong Sorn, Phayao, Nan, Lampang, Phrae, Lamphun, Uttharadith, Sukhothai, Tak, Phitsanulok, Phetchabun, Kamphaeng Phet, Nakorn Sawan, Uthai Thani, Sakon Nakorn, Nong Bua Lam phu, Chaiyaphum, Nakorn Ratchasima, Saraburi, Lop Buri, Kanchanaburi, Phrachin Buri, Ratchaburi, Phetchaburi, Prachuap Khiri Khan and Chumphon (Cherdshewasart *et al.*, 2006, in preparation).

The tuberous roots of the wild *B. superba* were collected from 25 provinces of Thailand, including Chiang Rai, Chiang Mai, Mae Hong Sorn, Phayao, Nan, Lampang, Phrae, Uttharadith, Tak, Phitsanulok, Phetchabun, Kamphaeng Phet, Nakorn Sawan, Uthai Thani, Sakon Nakorn, Loei, Nong Bua Lam phu, Khon Kaen, Chaiyaphum, Nakorn Ratchasima, Srisaket, Kanchanaburi, Lop Buri, Saraburi, Ratchaburi, Phrachin Buri, Chachoengsao, Chonburi and Chantaburi (Cherdshewasart, unpublished)

The tuberous roots of the wild *M. collettii* were collected from 4 provinces of Thailand, including Chiang Rai, Chiang Mai, Lumpang, Kanchanaburi (Cherdshewasart, unpublished).

3.1.2 Plant crude extraction (Cherdshewasart *et al.*, 2004[a])

The plant powder was extracted with absolute ethanol (Merck, Germany) by incubating 10 g of plant powder with 100 ml absolute ethanol for 4 days. The supernatants were filtered through filter paper (Whatman filterpaper No.1, Whatman, USA) and subsequently evaporated in the rotary evaporator N-1000 (EYELA, Japan) until completely dried. The crude extracts were stored in light-protect bottle at 4 °C until analysis.

The plant extracts were dissolved in DMSO and then filtered through a 0.45 μm sterile membrane filter disc (Minisart RC15, Sartorius, Germany) and assessed at

the concentration of 0.625, 1.25 and 2.50 mg/plate for mutagenicity and anti-mutagenicity assay. The concentrations were without toxicity to revertant bacterial colonies.

3.2 Bacterial strains

Salmonella Typhimurium stains TA98 (*hisD3052*, *rfa*, *uvrB*, pKM101) and TA100 (*hisG46*, *rfa*,*uvrB*, pKM101) were kindly provided by The Biochemistry and Chemical Carcinogenesis Section, Research Division, National Cancer Institute, Ministry of Public Health, Thailand. The tester strains obtained were primarily checked for their characteristics as described in Appendix A.

Bacillus subtilis strains H17, M45 were recombination-proficient (rec^+) and recombination-deficient (rec^-) respectively which were gifted from Dr. Malin Julsiri, Department of Microbiology, Faculty of Pharmacy, Mahidol University, Thailand.

3.3 Chemical and reagents

The chemicals and solvents used throughout the experiment were analytical grade. Glucose-6-phosphase (G-6-P), β-Nicotinamide adenine dinucleotide phosphate (NADP), d-Biotin (Vitamin H), L-Histidine, Sodium phenobarbital, 5,6-benzoflavone were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). Dimethysulfoxide (DMSO), sodium chloride, di-sodium hydrogen phosphate, sodium dihydrogen phosphate, citric acid monohydrate, potassium chloride, di-potassium hydrogen phosphate, D(+)glucose, sodium ammonium hydrogen phosphate, potassium dihydrogen phosphate, absolute methanol, magnesium sulfate and magnesium chloride were purchased from Merck (Dermstadt, Germany). Benzo(*a*)pyrene (B*(a)*P) was purchased from Sigma Chemical Company (St. Louis, Missouri, USA). 2-(2-Furyl)-3-(5-nitro-2-furyl)- acrylamide (AF-2) was purchased from Wako Pure Chemical Industrial, Ltd (Osaka, Japan). Bacto agar was purchased from Difco Laboratories (Detroit, Michigan, USA). Nutrient broth No.2 was purchased from Oxoid, Ltd (Basingstoke and Hants, England). Ampicillin was purchased from General Drug House Co, Ltd (Bangkok, Thailand). Barium chloride, Copper (II) sulfate pentahydrate, Sodium carbonate, Sodium carbonate, Sodium hydroxide, Sodium potassium tartrate, Folin-Ciocateu's phenol reagent and sulfuric acid were purchased from Merck (Dermstadt, Germany). Bacto tryptic soy broth was purchased from Difco Laboratories (Detroit, Michigan, USA).

3.4 Mammalian liver enzyme preparation

Male Sprague-Dawley rats, weighting 160-200 g B.W., were supplied by the National Animal Production Center, Mahidol University, Salaya Campus, Nakornpathum, Thailand,. The animals were kept in a control-room with a 12 hours light and dark cycle at temperature of 25 ± 3 °C. Animal feed and tap water was provided. Procedures for the S9 fraction have been described in Appendix A.

3.5 Experimental protocol

3.5.1 Reverse mutation assay (The Ames Salmonella/microsome assay, Maron and Ames, 1983)

The plant extracts were assayed in two systems, with or without metabolic activation. The experiment was divided in to three experiments.

3.5.1.1 Preparation of bacterial cultures

The bacteria in each strain from the frozen master copies was thawed in the water bath at 37°C and 10 µl of the thawed one was added to 12 ml of Oxoid Nutrient Broth No.2 in the flask and shaked at 120 rpm in a shaking water bath for 14 hours. Finally, the broth culture containing 1-2x10^9 CFU/ml was used for mutagenicity and anti-mutagenicity assay.

3.5.1.2 Preparation of nutrient agar

3.5.1.2.1 Minimal agar plate

30 ml of sterile minimal glucose agar medium containing 1.5% Bacto agar, 40% glucose, and 50x Vogel Boner salt was transferred to a sterile Petri dish. The recipe was described in Appendix A.

3.5.1.2.2 Top agar

Top agar containing 0.6% Agar and 0.5% NaCl was autoclaved and stored at room temperature. The agar was melted in a steam bath prior to assay. Ten ml of sterile solution containing 0.5 mM each of L-histidine and d-biotin were added to each 100 ml of the molten agar and mixed thoroughly by swirling (see the recipe in Appendix A).

3.5.1.3 Preparation of S9 mix for Mutagenicity and Antimutagenicity assay

3.5.1.3.1 S9 mix

Rat liver is the general source of activating enzyme. It is the efficient detection of a wide variety of carcinogens requiring metabolic activation.

The S9 fraction at -80°C was thawed at room temperature. 10 ml S9 mix composed of the ingredients; 6.6 ml, 0.2 M phosphate buffer pH 7.4, 0.4 ml, 0.1 M NADP solution, 0.5 ml, 1 M G-6-P solution, 0.5 ml, 0.16 M $MgCl_2$ solutions and 2 ml S9 fraction (see Appendix A). The S9 mix was freshly prepared and kept on ice during experiment.

3.5.1.4 Experiment I - Survival test

The test compound was further evaluated for toxicity such that the killing of the *His*$^+$ cells in the inoculum by the bactericidal and /or mutagenic compounds is measured under the conditions which are nearly indentical to those of Ames mutagenesis or anti-mutagenesis plate assay if the test compound showed a positive inhibitory response was in the range of 40-60%. Briefly described, 15-20 revertant conlonies of each *S.* Typhimurium strain TA98 and TA100 induced by AF-2 were isolated from the plate and inoculated into the sterile tube containing 0.2 M phosphate buffer pH 7.4. Each strain mixture was diluted with 0.2 M phosphate buffer and adjusted to give a density equivalent to Mc. Farland No.0.5 was approximately 10^8 CFU/ml (see Appendix B). The mixture was diluted 1:10,000 and 0.05 ml of the diluted mixture were used to replace mutagen were transferred into a sterile tube containing 0.5 ml 0.2 M phosphate pH 7.4, then 0.1 ml of crude extracts at the concentration of 2.5, 5,10 and 20 mg/plate, which had been shaken at 37 °C for 14 hours, was added into culture tube. Mixed gently and incubated in a shaking water bath at 37 °C for 20 min. The 2.5 ml of molten top agar containing 0.05 mM each of L-histidine and d-biotin were added, mixed gently and poured onto a minimal glucose agar plate. The *His*$^+$ revertant conlonies were counted after incubation at 37 °C for 48 hours. Each plant extract concentration was assayed in triplicate with the absence metabolic activation. Toxicological effects were determined by the growth inhibition of the background lawn under a light microscope.

3.5.1.5 Experiment II - Mutagenic activity of the plant extracts toward *S.* Typhimurium TA98 and TA 100 in the absence and presence of metabolic activation.

The experiment was illustrated in Figure 3.1. Briefly described, 0.1 ml of various concentrations of crude extracts was transferred into a sterile tube containing 0.5 ml 0.2 M phosphate pH 7.4 or S9 mix. Then 0.1 ml of the fresh broth bacterial culture, which had been shaken at 37 °C for 14 hours, was added into culture tube. Mixed gently and incubated in a shaking water bath at 37 °C for 20 min. Then 2.5 ml of molten top agar containing 0.05 mM each of L-histidine and D-biotin were added, mixed gently and poured onto a minimal glucose agar plate. The *His*$^+$ revertant conlonies were counted after incubation at 37 °C for 48 hours. Each concentration was assayed in triplicate with the absence and presence of S9 mix.

The positive and negative controls were included in each test. The negative control was 0.1 ml of DMSO and positive control in the absence of S9 mix was 0.1 ml of 0.1, 0.01 μg/plate AF-2 for TA98 and TA100 respectively while as that in the presence of S9 mix was 0.1 ml of 10,5 μg/plate B(*a*)P for TA98 and TA100 respectively. Toxicity effects of compounds on tester strains were determined by the growth inhibition of the background lawn under a light microscope.

3.5.1.6 Experiment III - Antimutagenic activity of the plant extracts toward *S.* Typhimurium TA98 and TA 100 in the absence and presence of metabolic activation.

The experiment III was illustrated in Figure 3.1. The 0.05 ml of various concentrations of crude extracts were transferred into a sterile tube containing 0.05 ml of standard mutagen, 0.5 ml of 0.2 M phosphate pH 7.4 or S9 mix and 0.1 ml of fresh broth bacterial culture, which had been shaken at 37°C for 14 hours. They were mixed gently and incubated in a shaking water bath at 37 °C for 20 min. The 2 ml of molten top agar containing 0.05 mM each of L-histidine and D-biotin were added, mixed gently and poured onto a minimal glucose agar plate. The *His*$^+$ revertant colonies were counted after incubation at 37 °C for 48 hours. The positive and negative controls were also incubated the same as previous described in the experiment II.

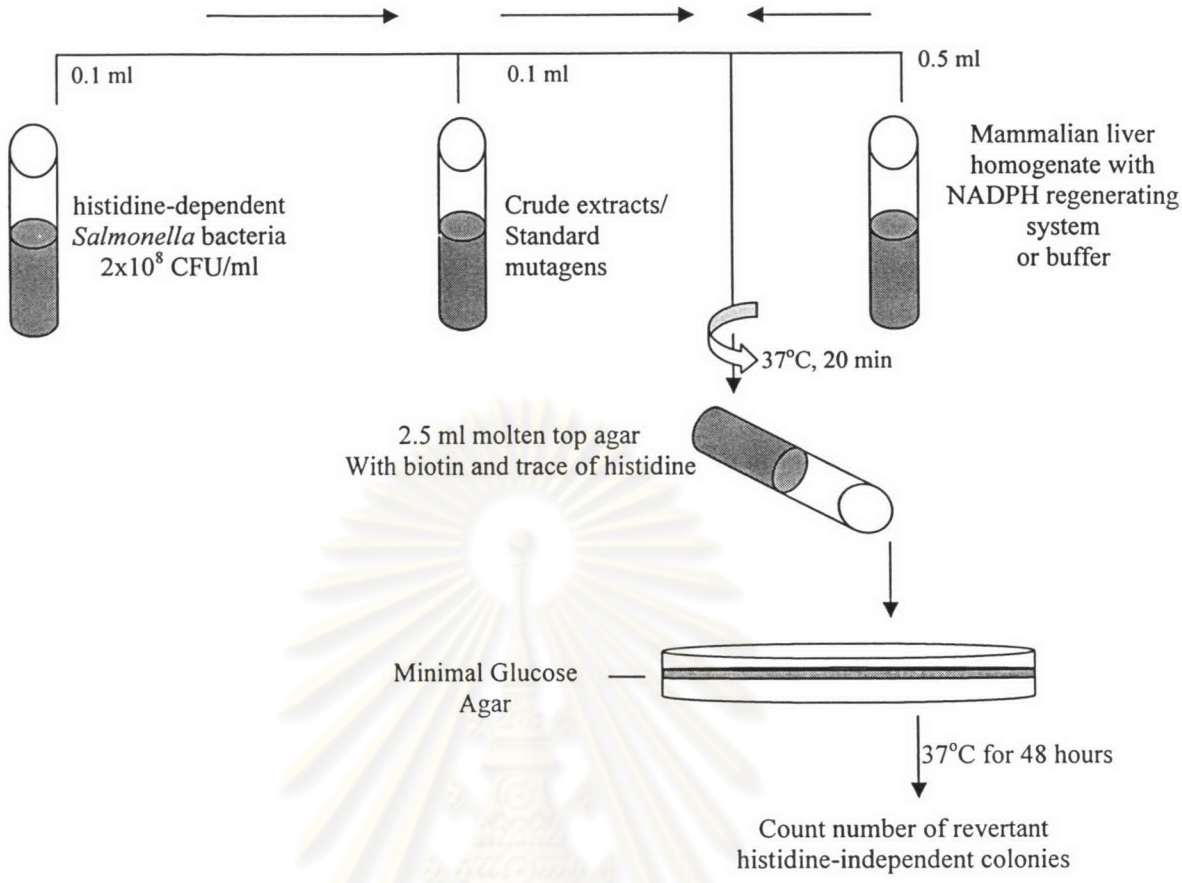

Figure 3.1 Diagram depicting the steps involved in the preincubation assay.

3.5.1.7 Interpretation

Mutagenicity assay, the number of revertants colonies induced by AF-2 and B(*a*)P mutagens were set as 100% mutagenicity, which has to be at least two of the number of spontaneous revertant colonies. Criteria of measurement for the mutagenicity revealed that the revertant colonies of the samples increase as two fold of the spontaneous revertant colonies, samples was dissolved at the highest of the concentration or until the cytoxicity was found.

For antimutagenicity assay, the revertant colonies obtained were calculated for Percent Inhibition (PI) of mutagenicity by plant extracts, using the following formula (Ong *et al.*,1986)

$$PI = (1-(N_t / N_o) \times 100$$

N_t is the number of *His*$^+$ revertants per plate in the presence of plant extract.

N_o is the number of *His*$^+$ revertants per plate in the absence of plant extract.

The potential of antimutagenic effect was considered strong when the inhibitory effect was higher than 60% inhibition and moderate when the inhibitory effect was in the range 40-60%. Inhibitory effect less than 40% were considered weak, and it was not recognized as a positive result (Ikken, Camberol and Marrin *et al.*,1998).

3.5.1.8 Statistical analysis

The results of all tested parameters were expressed as mean ± S.E.M. of three independent experiments. Analysis of variance (ANOVA) was used to determine the differences of means using the Statistical Packages for Social Science (SPSS) version 10.0 in all of the parameters. The observed significance was then confirmed using the least significance difference (LSD) test. Statistical significance were defined as $P < 0.05$, when statistically significant inter group differences were identified by ANOVA and post hoc analyses were performed using a Duncan's multiple range test.

3.5.2 Forward mutation assay

The method of *Rec* assay system described by Kada, 1980. The plant crude extracts were assayed without metabolic activation. The experiment was divided into two experiments.

3.5.2.1 Preparation of plant materials

Plant extracts with the highest antioxidant activity (Sutjit, 2003), *P. lobota* (IC_{50} of 2,482.00 ± 66.11 μg/ml), *P. mirifica* from Uthai Thani (IC_{50} of 2,904.52 ± 33.24 μg/ml), *B. superba* from Loei (IC_{50} of 653.64 ± 38.83 μg/ml) and *M. collettii* from Chiang Rai (IC_{50} of 83.06 ± 8.10 μg/ml) were chosen for mutagenic and antimutgenic studies by *Rec* assay. Plant extracts were assessed at the concentrations of 2.5, 5 and 10 mg/plate.

3.5.2.2 Preparation of cultures

The well-isolated spore suspension of *B. subtilis* strains H17(rec^+) and M45 (rec^-) were incubated into tryptic soy broth, then incubated in a shanking waterbath at 37°C for 18 hours. Finally, the broth culture was adjusted to a cell density that equivalent to Mc.Farland No.2 (see Appendix B), approximately $2x10^7$ spores/ml.

3.5.2.3 Experiment I - Mutagenic activity of the plant extracts toward *B. subtilis* H17 (rec^+) and M45 (rec^-) in the absence of metabolic activation.

The rec^- assays were carried out with spores of *B. subtilis* strains H17 and M45. Spore-agar plates were prepared by mixing with 0.1 ml of spore suspension ($2x10^7$ spores/ml) and 10 ml of tryptic soy agar. After the agar was solidified, it was pierced with sterile cork borer of diameter 8 mm to obtain a hole, totally of 5 holes per plate. Each well was applied with 0.02 ml of DMSO and 0.02 ml of each sample solution. After overnight incubation at 37°C, the diameter of inhibition zones around the well for H17 and M45 spore plates were measured and compared. Each concentration was assayed in triplicate. The negative control was 0.04 ml of DMSO while that positive control was 0.02 ml of 5 μg/ml of AF-2 and 0.02 ml of DMSO for both H17 and M45, respectively.

3.5.2.4 Experiment II - Antimutagenic activity of the plant extracts toward *B. subtilis* H17 (*rec*$^+$) and M45 (*rec*$^-$) in the absence of metabolic activation.

Preparation of the agar with *B. subtilis* in the Petri dish was described in phase I. Each well was applied with 0.02 ml 5 μg/ml of AF-2 and 0.02 ml of each sample solution. After overnight incubation, the diameters of inhibition zones around the well for H17 and M45 spore plates were measured. The positive and negative controls were included in each set by overnight incubation at 37°C as previous describe in experiment I.

3.5.2.5 Interpretation

The result was interpreted as positive for mutagenic effect when the M45/H17 ratio of the clear zone was greater of standard mutagen, while antimutagenic effect if M45/H17 ration of inhibition zones was less than standard mutagen as sample solution has antimutagenicity.

3.5.2.6 Statistical analysis

The results were expressed as mean ± S.E.M. of three independent experiments. Unpaired Student's T-test was used for analysis of the test results and $P < 0.05$ was considered significant. The software used was SPSS version 10.0 statistical software program.