CHAPTER III

MATERIALS AND METHODS

3.1 Plant materials and extraction

3.1.1 Plant materials (Cherdshewasart unpublished)

P. mirifica tubers were collected from 28 provinces, including Uthai Thani, Nong Bua Lam Phu, Phetchaburi, Phitsanulok, Ratchaburi, Chiang Mai, Nakhon Sawan, Lumphun, Nan, Chiang Rai, Sukhothai, Kanchanaburi, Lop Buri, Phrae, Chumphon, Nakhon Ratchasima, Sakon Nakhon, Phayoa, Lampang, Uttharadith, Tak, Chaiyaphum, Mae Hong Son, Prachuap Khiri Khan, Phrachin Buri, Saraburi and Kamphaeng Phet provinces.

P. lobata (Kudzu) tubers were collected from Ghangzhou, China.

B. superba tubers were collected from 23 provinces including Loei, Lop Buri, Saraburi, Lampang, Uttharadith, Phetchabun, Chonburi, Sakon Nakhon, Phitsanulok, Chiang Mai, Tak, Ratchaburi, Chiang Rai, Chantaburi, Kanchanaburi, Chaiyaphum, Chachoengsao, Srisaket, Prachinburi, Mae Hong Son, Nong Bua Lam Phu, Khon Kaen and Nakhon Sawan provinces.

M. collettii stems were collected from 4 provinces including Chiang Rai, Chiang Mai, Lampang and Kanchanaburi provinces.

The plant materials were sliced, dried in a hot air oven at 70 °C, 3 hours and subsequently ground into powder at a size of 100 Mesh.

3.1.2 Crude extraction preparation

The plant powders were extracted as follows. Fifty gm of the powder were dissolved in 500 ml ethanol for 1 week. The supernatants were filtered through Whatman filters No.1. The filtrates were evaporated in a rotary evaporator until completely dried and kept at 4 °C. The weights of the crude extracts were recorded (Table 3.1).

Table 3.1 The weights of the crude extracts in ethanol

Crude extracts						
No.	***P. mirifica***		***B. superba***		***M. collettii***	
	Sources (provinces)	**Weight (g)**	**Sources (provinces)**	**Weight (g)**	**Sources (provinces)**	**Weight (g)**
1	Uthai Thani	2.00	Loei	2.82	Chiang Rai	3.19
2	Nongbua Lamphu	2.13	Lop Buri	2.33	Chiang Mai	2.89
3	Phetchaburi	2.29	Saraburi	2.18	Kanchanaburi	2.68
4	Phitsanulok	2.38	Lampang	2.64	Lampang	2.06
5	Phetchabun	3.24	Uttharadith	2.22		
6	Ratchaburi	2.43	Phetchabun	2.70	**Sources (provinces)**	**Weight (g)**
7	Chiang Mai	1.88	Chonburi	3.69	***P. lobata***	1.16
8	Nakhon Sawan	3.01	Sakon Nakhon	2.07		
9	Lamphun	2.37	Phitsanulok	3.79		
10	Nan	1.39	Chiang Mai	3.87		
11	Chiang Rai	3.84	Tak	2.22		
12	Sukhothai	2.2	Ratchaburi	2.60		
13	Kanchanaburi	1.27	Chiang Rai	2.67		
14	Lopburi	2.85	Chantaburi	2.67		
15	Phrae	1.13	Kanchanaburi	2.61		
16	Chumphon	1.97	Chaiyaphum	1.29		
17	Nakhon Ratchasima	2.82	Chachoengsoa	3.83		
18	Sakon Nakhon	3.22	Srisaket	2.74		
19	Phayoa	1.85	Phrachinburi	2.30		
20	Uttaradit	2.17	Mae Hong Son	2.32		
21	Lampang	3.75	Nong Bua Lam Phu	2.98		
22	Tak	2.45	Khon kaen	3.06		
23	Chaiyaphum	2.03	Nakhon Sawan	2.70		
24	Mae Hong Son	2.31				
25	Prachuap Khiri Khan	3.96				
26	Prachinburi	1.38				
27	Saraburi	2.65				
28	Kamphaeng Phet	2.68				

3.2 Antioxidant activity test

The antioxidant activity was carried out according to procedure with modification. This reaction has been widely used to test the ability of compounds to be free-radical scavengers or a hydrogen donor of plant extracts. The method is based on the reduction of DPPH, a stable free radical. Because of the odd electron of DPPH, it gives a strong absorption maximum at 517 nm by visible spectroscopy (purple color). As the odd electron of the radical becomes paired off that is antioxidant activity, which the absorption strength is decreased, and the resulting depolarization is stoichiometric with respect to the number of electrons captured (Blois *et al.*, 1958). Incubation with the extracts resulted in decrease of the absorbancy with a color change from purple to yellow as the radicals are scavenged by antioxidant through donation of hydrogen to from the stable DPPH-H molecule *in vitro* with α-tocopherol as positive control. The results were indicated as the concentration required effecting a 50% inhibition of decreasing signal peak height (IC_{50}) by microtiter plate reader

3.2.1 Preparation for antioxidant activity test

3.2.1.1 Preparation of plant samples

Six mg crude extracts each of *P. mirifica*, and *P. lobata*, 3 mg crude extract of *B. superba* and 1 mg crude extract of *M. collettii* were dissolved in 1 ml absolute ethanol for stock solution. One mg/ml in absolute ethanol α - tocopherol is adopted as a positive control. Puerarin, daidzin, genistin, daidzein and genistein were prepared at $10^{-4} - 10^{-9}$ M as positive controls for *P. mirifica* and *P. lobata*. Flavonoid and sterol fraction in ethanol (1 mg/ml) as positive controls for *B. superba*, which collected from Lampang province.

3.2.1.2 Chemicals and reagents

The stable free radical used was 1, 1-diphenyl-2-picrylhydrazyl (DPPH; Fluka). Other chemicals and applied reagent were absolute ethanol (Merck),

α - tocopherol (Vitamin E; Fluka Biochemika). Puerarin, daidzin, genistin, daidzein and genistein were purchased from Sigma, USA

3.2.1.3 Instrumentation

Microtiter plate reader (SUNPRISE-TECCAN ASTRIA GMBTL) was performed using for DPPH assay at 517 nm.

3.2.2 Experimental protocol

1) DPPH solution was freshly prepared at 200 μM in absolute ethanol (0.0787 mg of DPPH in 125 ml absolute ethanol) and kept in the dark by cover with aluminum foil until used. The absorbance of 200 μl DPPH solution was measured at 517 nm for setting up of reagent blank.

2) Kwao Krua crude extracts (6 mg/ml of *P. mirifica* as same as *P. lobata*, 3 mg/ml of *B. superba* and 1 mg/ml of *M. collettii*) and α-tocopherol (1 mg/ml, in a total volume of 1 ml) in absolute ethanol as stock solution were diluted.

3) Serial amount of α-tocopherol of 0, 3.12, 6.25, 12.5, 25, 37.5 and 50 μg/ml ethanol were pipetted into Eppendorf tube and protect from light with aluminum foil. The screening test of *P. mirifica* and *P. lobata* stock solutions were firstly diluted with absolute ethanol to establish the concentration of 0, 12.5, 25, 50, 100, 250, 500, 1,000 and 1,500 μg/ml, in aliquot volume of 50 μl. *P. mirifica* and *P. lobta* crude extracts were chosen at the concentration of 75, 112.5, 150, 187.5, 225 and 300 μg/ml for the final test. *B. superba* stock solution was diluted to 56.25, 75, 93.75, 112.5, 131.25 and 150 μg/ml ethanol in aliquot volume of 50 μl. *M. collettii* stock solution was diluted to 20, 40, 60, 80, 100 and 200 μg/ml ethanol in an aliquot volume of 50 μl.

4) DPPH solution, 950 μl and 50 μl sample solution or tocopherol was added into each tube to establish 1 ml final volume.

5) The mixture was shaken vigorously and incubated at 37 °c in the dark at room temperature for 30 minute.

6) Two hundred μl of the mixed solution was transferred to a 96–well microtiter plate. The absorbances of the samples were measured at 517 nm against

DPPH blank. Measurements were performed in triplicate in three independent experiments.

3.2.3 Interpretation

Data were analyzed by SPSS program. The antioxidant activity is favorable express in 2 criteria, IC_{50} value and PI value. The results revealed that the IC_{50} value and PI value of the plant extracts showed slightly different in the same test due to the fact that different concentration range were set up but the calculation for PI had to based on a certain concentration not the whole concentration range as did integrated by the calculation for IC_{50}. In this study, the IC_{50} was chosen for discussion of the antioxidant activity of DPPH assay. The fifty percent inhibition (IC_{50}) of each sample was determined.

3.3 Mutagenicity and antimutagenicity by Ames' test (preincubate technique)

Of the numerous short-term genotoxicity assays, an *in vitro* microbial mutagenicity assay developed by Bruce Ames (Ames and Maron, 1983) has become the most widely used and most thoroughly investigated. The assay is rapid and inexpensive. The Ames assay is a well-known assay for the detection of mutagens as well as antimutagens.

3.3.1 Preparation of the mutagenicity and antimutagenicity test

3.3.1.1 Preparation of plant samples

P. mirifica collected from Uthai Thani, *B. superba* collected from Loei, and *M. collettii* collected from Chiang Rai together with *P. lobata*, in antioxidant test were chosen for mutagenic and antimutagenic studies by Ames' test. Plant extracts were assessed at the concentration of 2.5, 5, 10 and 20 mg/plate for mutagenicity assay. The concentrations without toxicity to revertant bacterial colonies were chosen for antimutagenicity test.

3.3.1.2 Chemicals and reagents

All chemicals and solvents used throughout the experiment were analytical grade. Glucose-6-phosphate (G-6-P), β-Nicotinamide adenine dinucleotide phosphate (NADP), d-Biotin, L-Histidine, sodium phenobarbital, 5, 6-benzoflavone were purchased from Sigma Chemical Company, St. Louis, Missouri, USA: Dimethylsulfoxide, 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, Darmstadt, Germany. Benzo[a]pyrene (B(a)P) was purchased from TCI-EP Co., Tokyo, Japan. AF_2 was purchased from Sigma Chemical Co., USA. Bacto agar was purchased from Difco Laboratories, Detroit, Michigan, USA. Nutrient broth No.2 was purchased from

Oxoid Ltd., Basingstoke and Hants, England. Normal saline 0.85% and ampicillin was purchased from General Drugs House Co. Ltd., Bangkok, Thailand.

3.3.1.3 Bacterial strains

S. Typhimurium TA 98 (*hisD3052*, r*fa*, uvrB, pKM101) and TA 100 (*hisG46*, r*fa*, uvrB, pKM101) strains were kindly provided by Department of Tropical Nutrition and Food Science, Faculty of Tropical Medicine, Mahidol University. S. Typhimurium TA 98 and TA 100 were used as standard tester strains for mutagenicity and antimutagenicity test from other a set of histidine requiring stains (see Appendix II). Each tester strain contains a different type of mutation in the histidins operon; S. Typhimurium TA 98 and TA 100 were histidine-dependent strains (His^-) are capable of detecting frameshift mutation and base-pair substitute mutation, respectively. The tester strains obtained were primarily checked for their characteristics as described in Appendix II.

3.3.1.4 Animal for enzyme preparation

Male Sprague-Dawley rats, weighing 160-200g B.W., supplied by the National Animal Production Center, Mahidol University, Salaya Campus, Nakornpathom, Thailand, were used in the experiments. All animals were kept in a room with a 12 hours light and dark cycle at temperature of 25±3 °C. Animal feed and tap water is provided.

3.3.2 Experimental protocol

3.3.2.1 Preparation of S. Typhimurium TA98 and TA100

1.) The bacteria 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 shaking water bath at 37 °C for 14 hours.

2.) The mixture of 1 ml fresh overnight culture and 7 ml of 0.9 percent normal saline was measured at 620 nm, using spectrophotometer. This culture of bacteria

was used in the Ames'test at a density of 1-2x10^9 cells per ml or equivalent to optical density (OD.) of 0.3-0.4.

3.3.2.2 Preparation of liver S9 fraction and S9 mix

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

3.3.2.2.1 Induction of mammalian liver enzymes

The rats were injected intraperitoneally (i.p) with sodium phenobarbital and 5, 6-benzoflavone for induction of liver drug metabolizing enzymes. The induction procedure was described as follows (Matsushima *et al.*, 1976):

1) A single (i.p) injection of 10 mg/ml of sodium phenobarbital in saline solution, at a dosage of 30 mg/kg B.W. in the morning of the first day.

2) A single (i.p) injection of 20 mg/ml of sodium phenobarbital in saline solution, at a dosage of 60 mg/kg B.W. in the morning of the second day.

3) A single (i.p) injection of 20 mg/ml of sodium phenobarbital in saline solution, at a dosage of 60 mg/kg B.W. in the morning as second day and a single (i.p) injection of 10 mg/ml of 5,6-benzoflavone in corn oil at a dosage of 80 mg/kg B.W. in the afternoon of the third day.

4) A single (i.p) injection of 20 mg/ml of sodium phenobarbital in saline solution, at a dosage of 60 mg/kg B.W. in the morning of the fourth day as second day, and the animal are given drinking water adlibitum until 12 hours before sacrificed

3.3.2.2.2 Removal of liver

All surgical instruments, glasses and solution were sterilized ones; the rats were killed by cervical dislocation on the fifth day. The livers must be removed with sterile technique, as followed:

1) Flooded the fur around the abdominal with 70% ethanol and cut through skin with sterile scissors.

2) Swabbed the muscle layer with 70% ethanol and then cut through it with a fresh pair of sterile scissors.

3) The livers were removed by cutting through the blood vessels and connective tissue.

3.3.2.2.3 Liver homogenate S9 fraction (Garner *et al.*, 1972)

All steps of the procedure were carried at 0-4 °C using cold solutions. The freshly excised livers were washed several times with cold 0.15 M KCl solution and then the livers were weighed. The washed livers were transferred to a beaker containing 3 volumes of a cold 0.15 M KCl (3 ml/g of wet livers) solution for several times (KCl solution is essential to insure a sterile preparation and to remove hemoglobin, which can inhibit the activity of the cytochrome P450 enzymes). The livers were chopped with a sterile scissors and homogenized in a Potter-Elvehjem apparatus with a Teflon pestle. The homogenate was centrifuged for 20 minutes at 9000 g at 4 °C for 10 min. The supernatant (S9 fraction) were decanted immediately and distributed in 1-2 portion at –80 °C for used. The sterility of S9 fraction was determined by plating 0.1 ml on minimal glucose agar plate containing histidine and biotin. There should be no growth of bacteria on the plates.

3.3.2.2.4 Preparation of S9 mix for mutation assay

The S9 fraction at – 80 °C was thawed at room temperature. Ten ml S9 mix composed of the ingredients: 6.6 ml, 0.2 M phosphate buffer pH 7.4, 0.4 ml, 0.1 M NADPH solution, 0.5 ml, 1 M G6P solution, 0.5 ml, 0.16 $MgCl_2$ solutions and 2 ml S9 fraction (see Appendix II). The S9 mix was freshly prepared and kept in ice box during experiment.

3.3.2.3 Reversion mutation assay (Ames'test: preincubation method) (Ames and Maron, 1983)

Ames' test was performed to test for the mutagenic activity (Table 3.2-3.3) and antimutagenicity (Table 3.4-3.5) of Kwao krua plant extracts, *P. mirifica*, *P. lobata*, *B. superba* and *M. collettii* and each crude extract was tested in two systems, with or without metabolic activation. Four concentrations of the samples at 2.5, 5, 10 and 20 mg/plate were tested by preincubation method.

3.3.2.3.1 Testing for mutagenicity

Table 3.2 Experimental designs for mutagenicity test of TA98 strain

Groups	Treatment	Preincubation mixture
I (-S9 mix, TA98 strain)	Mutagenicity test	50 µl sample + 50 µl DMSO + 500 µl phosphate buffer pH 7.4 + 100 µl TA98
	Positive control	50 µl mutagen (AF_2) + 50 µl DMSO +500 µl phosphate buffer pH 7.4 + 100 µl TA98
	Negative control	50 µl water + 50 µl DMSO +500 µl phosphate buffer pH 7.4 + 100 µl TA98
II (+S9 mix, TA98 strain)	Mutagenicity test	50 µl sample + 50 µl DMSO +500 µl S9 mix + 100 µl TA98
	Positive control	50 µl mutagen (B(a)P)+ 50 µl DMSO + 500 µl S9 mix + 100 µl TA98
	Negatives control	50 µl water + 50 µl DMSO + 500 µl S9 mix + 100 µl TA98

Remark: Groups I and II should be tested together for compared in either the absence enzymatic (-S9 mix) or the presence enzymatic (+S9 mix) of the tester strains

Table 3.3 Experimental designs for mutagenicity test of TA100 strain

Groups	Treatment	Preincubation mixture
I (-S9 mix, TA100 strain)	Mutagenicity test	50 µl sample + 50 µl DMSO + 500 µl phosphate buffer pH 7.4 + 100 µl TA100
	Positive control	50 µl mutagen (AF_2) + 50 µl DMSO +500 µl phosphate buffer pH 7.4 + 100 µl TA100
	Negative control	50 µl water + 50 µl DMSO +500 µl phosphate buffer pH 7.4 + 100 µl TA100
II (+S9 mix, TA100 strain)	Mutagenicity test	50 µl sample + 50 µl DMSO +500 µl S9 mix + 100 µl TA100
	Positive control	50 µl mutagen (B(a)P) + 50 µl DMSO + 500 µl S9 mix + 100 µl TA100
	Negatives control	50 µl water + 50 µl DMSO + 500 µl S9 mix + 100 µl TA100

Remark: Groups I and II should be tested together for compared in either the absence enzymatic (-S9 mix) or the presence enzymatic (+S9 mix) of the tester strains

3.3.2.3.2 Testing for the antimutagenicity

Table 3.4 Experimental designs for the antimutagenicity test in TA98 strain

Groups	Treatment	Preincubation mixture
I (-S9 mix, TA98 strain)	Antimutagenicity test	50 µl sample + 50 µl mutagen (AF_2) + 500 µl phosphate buffer pH 7.4 + 100 µl TA98
	Positive control	50 µl mutagen (AF_2) + 50 µl DMSO + 500 µl phosphate buffer pH 7.4 + 100 µl TA98
	Negative control	50 µl water + 50 µl DMSO + 500 µl phosphate buffer pH 7.4 + 100 µl TA98
II (+S9 mix, TA98 strain)	Antimutagenicity test	50 µl sample + 50 µl mutagen (B(a)P) + 500 µl S9 mix + 100 µl TA98
	Positive control	50 µl mutagen (B(a)P) + 50 µl DMSO + 500 µl S9 mix + 100 µl TA98
	Negatives control	50 µl water + 50 µl DMSO + 500 µl S9 mix + 100 µl TA98

Remark: Groups I and II should be tested together for compared in either the absence enzymatic (-S9 mix) or the presence enzymatic (+S9 mix) of the tester strains

Table 3.5 Experimental designs for the antimutagenicity test in TA100 strain

Groups	Treatment	Preincubation mixture
I (-S9 mix, TA100 strain)	Antimutagenicity test	50 µl sample + 50 µl mutagen (AF_2) + 500 µl phosphate buffer pH 7.4 + 100 µl TA100
	Positive control	50 µl mutagen (AF_2) + 50 µl DMSO + 500 µl phosphate buffer pH 7.4 + 100 µl TA100
	Negative control	50 µl water + 50 µl DMSO + 500 µl phosphate buffer pH 7.4 + 100 µl TA100
II (+S9 mix, TA100 strain)	Antimutagenicity test	50 µl sample + 50 µl mutagen (B(a)P) + 500 µl S9 mix + 100 µl TA100
	Positive control	50 µl mutagen (B(a)P) + 50 µl DMSO + 500 µl S9 mix + 100 µl TA100
	Negatives control	50 µl water + 50 µl DMSO + 500 µl S9 mix + 100 µl TA100

Remark: Groups I and II should be tested together for compared in either the absence enzymatic (-S9 mix) or the presence enzymatic (+S9 mix) of the tester strains

The mixtures were mixed and incubated in shaking water bath at 37 °C for 20 min. After incubation, 2 ml of molten top agar at 45 °C containing 0.5 mM of histidine-bitin solution (ratio of amino acid: agar = 1:10) was added. The mixture was mixed well before pouring onto a minimal glucose agar plates (see Appendix II). Plates were incubated at 37 °C, 48 hours. The histidine revertant colonies were counted. Otherwise, inducing mutations, the test chemical may also result in bacterial toxicity, the background growth lawn of the tester strains may be reduce the density when compared to the lawn of the negative control. This was examined under a microscope (x 40 magnification), the lawn will be seen consist of very small spaced colonies. Each concentration of the plant extract was performed in triplicate with or without of S9 mix. The concentration mutagens were used in this experimental is shown in Table 3.6.

Table 3.6 The concentrations of the standard mutagens

Mutagens	**Concentration (µg/plate)**			
	TA 98		**TA 100**	
	+S9	**-S9**	**+S9**	**-S9**
AF_2	0.1	-	0.01	-
B(a)P	-	10	-	5

Remark: AF_2 = 2-[2-furyl]-3-[5-nitro-2-furyl]acrylamide
B(a)P = Benzo[a]pyrene

3.3.3 Interpretation

3.3.3.1 Mutagenicity test

The number of revertants colonies induced by AF_2 and B(a)P mutagens was 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 increased as two fold of the spontaneous revertant

colonies, samples was dissolved at the highest of the concentration or until the cytoxicity was found.

3.3.3.2 Antimutagenicity test

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 = Number of mutagen induced revertant colonies per plate in the presence of plant extract

N_o = Number of mutagen induced revertant colonies per plate in the absence of plant extract

The potential of antimutagenic effect of the test materials were interpreted according to scheme shown in Table 3.7

Table 3.7 The potential of antimutagenicity effect (Wall *et al.*, 1988)

PI	Ranking for antimutagenicity
0-20	Negative
20-40	Weak
40-60	Positive
60-90	Strong
>90	Toxicity suspected

3.3.3.3 Statistical evaluation

The statistical method, One Way ANOVA, was used to calculate for the means and Standard Error (S.E.) of mean.

3.4 Genotoxicity by micronucleus test

3.4.1 Preparation for micronucleus test

3.4.1.1 Preparation of plant samples

P. mirifica, *P. lobata*, *B. superba* and *M. collettii* crude extracts (equivalent to approximately LD_{50} value of *P. mirifica* was 16 g powder/kg. B. W. (Chivapat *et. al.*, 2000) from samples with highest antioxidant activity (IC_{50}) in antioxidant test was chosen for their mutagenic activity studies in mammalian model by micronucleus assay. They were dissolved in 1% absolute ethanol (1 ml) for setting up of stock solution.

3.4.1.2 Animals

Male Wistar rats, weighing 160-200g B.W., supplied by the National Animal Production Center, Mahidol University, Salaya Campus, Nakornpathom, Thailand, were used in the experiments. All animals were kept in a room with a 12 hours light and dark cycle at temperature of 25 ± 3 °C. Animal feed and tap water is provided for 1 week before the experiment date.

3.4.1.3 Chemicals and reagents

Chemicals used in the micronucleus test were analytical grade, namely, Absolute ethanol and Ethyl ether was purchased from E. Merck, Darmstadt, Germany. May-Grunwald's stain, Giemsa's stain was purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Cyclophosphamide was purchased from Asta Medica, Frankfurt, Germany. Fetal calf serum was purchased from Gibco Laboratories, New York, USA.

3.4.2 Experimental protocol

3.4.2.1 Experimental design

Micronucleus arises in mitotic cells from chromosomal fragment or chromosomes that lag behind in anaphase. It is used for the study of the effect of chromosome damage in rat's bone marrow. Plant crude extract solution was fed to animals by the stomach tube in the volume 0.3-0.7 ml. Each of mutagen was injected (i.p.) in the volume of 0.3-0.7 ml depending on the animal body weight. The negative control was 1% absolute ethanol (Heddle *et al.*, 1983).

3.4.2.1.1 Dose variation study

The experimental design was recommended to find out the optimal dose of test compounds to induce the micronucleus, in order to save the animal and time. In dose variation study, at 0.1, 1 and 10 g/kg B. W. of the plant extract were administered to the animals; 80 mg/kg B. W. of CP was injected (i.p.) and negative controls were treated with solvent only (3 male rats for each dose). They were killed at 30 hours post-treatment and bone marrow cells of these rats were collected (As chemical mutagen showed that the maximum of micronuclei appeared at 30-36 hours after treatment). The dose which induced a significantly increase in the frequency of MNPCEs compared to negative control was used as the optimal dose, if non of all dose could induced this effect, the highest dose would be chosen in the time course effect.

3.4.2.1.2 Time variation study

The time course effects of test compound at the optimal dose, the plant extracts were administered to the animals, and 80 mg/kg B. W. of Cyclophosphamide was injected i.p. and negative controls were treated with 1% absolute ethanol (6 male rats for each time). Their bone marrow cells were collected at 24, 48 and 72 hours after treatment.

3.4.2.2 Preparation of bone marrow cells

The treated rats were killed by deep anesthetized with ethyl ether. Femurs were removed, muscular tissues were cleaned and the bones were cut open and flushed with 1 ml fetal calf serum by 25 G needle.

3.4.2.3 Bone marrow smears

After repeated aspiration and flushing, the collected fluids were centrifuged in a centrifuge for 5 min at 1,000 rpm. The supernatant was discarded with the Pasteur pipette and the bone marrow was mixed with a few drops of fetal calf serum. The cells suspension were dropped on the end of a dry and clean glass slide and were spread by pulling the droplet of cells behind another slide of glass which was held at angle of 45 ^{O}C. The slides were left air-dried for about 3 hours before staining.

3.4.2.4 Staining of bone marrow

1) The slides were fixed in absolute methanol for 20 min.

2) The slides were covered with May-Grunwald's stain which was undiluted with distilled water for 2 min.

3) The slides were covered again with May-Grunwald's stain, freshly diluted with distilled water 1:2, for 3 min.

4) The slides were covered with Giemsa's stain, freshly diluted with distilled water 1:6, for 10 min.

5) The slides were washed with tap water for 3 times and then air-dried at room temperature.

3.4.3 Interpretation

3.4.3.1 Microscopic analysis

The staining must allow clear discrimination between PCEs and NCEs. PCEs and NCEs should be purple and red, respectively. Several parameters must be set to identify cells, the number of PCEs, the area of the slides, the definition of size and

shape and color of cells and micronuclei to be scored. Cells were scanned for the presence of micronucleus by microscopic examination using a 100 x objective lens which was chosen for analysis of the well separated erythrocytes, clear between polychromatic erythrocytes (PCEs) and normachromatic erythrocytes (NCEs). The cytotoxicity was determined as the ratio of PCEs/NCEs in 1,000 erythrocytes per slide. If this ratio is ≤ 0.1, it indicates that the test compound has cytotoxic effect. The number of micronucleated PCEs (MNPCEs) in a total of 2,000 PCEs per slide was counted. The counted cells were expressed as the ratios of PCEs/NCEs in percent and MNPCEs/1,000 PCEs.

3.4.3.2 Statistical analysis

In the micronucleus test, statistical methods were used to calculate the means and standard error of mean. The mean frequencies of PCEs/NCEs and MNPCEs of sample extracts and negative control were statistically calculated using Student's t-test. Statistical significance of difference between groups was taken at p-value of less than 0.05.