CHAPTER III
MATERIALS AND METHODS

3.1 Plant materials

The fresh tuberous roots of *P. mirifica* and *B. superba* were sliced, dried in hot air oven at 70 °C and subsequently ground into powder at 100 Mesh size. The powder was kept in the desiccators under darkness until the preparations of suspension in distill water. The freshly prepared suspension was used.

3.2 Carcinogen

7,12-Dimethlybenz (a) anthracene (7,12-DMBA) (Sigma Chemical Company, U.S.A.) was used to induce mammary carcinoma in rats. The solution of 2% wt/vol of 7,12- DMBA in corn oil (Mazola, Thailand) was prepared with the aid of gentle heating at 100 °C for 30 minutes to aid the solubility. The fresh solution was used.

3.3 Experimental animals

Female Spargue-Dawley rats were supplied by National Laboratory Animal Center, Mahidol University. The rats were housed at the Animal's Laboratory House, Primate Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University. Five rats were housed in a stainless steel cage, 24.5x46x14 cm. of size, throughout an environment-controlled room with 25-26 °C and a 12-hour light/12-hour dark cycle. The animals were fed with rat chow diet (Pokapan Animal Food Center, Thailand) and tap water *ad libitum*.

3.4 Rat chow diet

Animal chow diet was purchased from Pokapan animal Food Centre, Thailand. Ingredients of the diet were shown in Table 3.1.

Table 3.1 Ingredients of the rat chow diet

Product specification		
Product name:	C.P.082	
Description:	Rat and mice food	
Ingredients:	Yellow corn, soybean meal, full-fat soybean, rice, rice by-product, fish meal, corn gluten meal, vegetable oil, salt, vitamins and minerals	
Nutrients:	Moisture	<10.0%
	Protein	>24.0%
	Fat	>4.5%
	Fiber	<5.0%
	Calcium	>0.9%
	Phosphorus	>0.7%

3.5 Experimental design

3.5.1 Experiment I: Evaluation of preventive activities of *P. mirifica* and *B. superba* in DMBA-induced rat mammary carcinoma

One hundred and forty female Spargue-Dawley rats, 23 day-old, 60 to 80 g body weight were selected. They were randomly divided into 7 groups (20 rats/group) as follows;

1.Control group: Rats were orally treated with 0.7 ml of distilled water per day for 4 consecutive weeks.

2. PM1 group: Rats were orally treated with the powder suspension of *P. mirifica* at the dosage of 10 mg/kg BW/ day in 0.7 ml of distilled water for 4 consecutive weeks.

3. PM2 group: Rats were orally treated with the powder suspension of *P. mirifica* at the dosage of 100 mg/kg BW/day in 0.7 ml of distilled water for 4 consecutive weeks.
4. PM3 group: Rats were orally treated with the powder suspension of *P. mirifica* at the dosage of 1000 mg/kg BW/day in 0.7 ml of distilled water for 4 consecutive weeks.
5. BS1 group: Rats were orally treated with the powder suspension of *B. superba* at the dosage of 10 mg/kg BW/day in 0.7 ml of distilled water for 4 consecutive weeks.
6. BS2 group: Rats were orally treated with the powder suspension of *B. superba* at the dosage of 100 mg/kg BW/day in 0.7 ml of distilled water for 4 consecutive weeks.
7. BS3 group: Rats were orally treated with the powder suspension of *B. superba* at the dosage of 1000 mg/kg BW/day in 0.7 ml of distilled water for 4 consecutive weeks.

After treatment, the rats were induced for mammary tumor by the single treatment of 80 mg/kg BW 7,12-DMBA. Following the exposure to 7,12-DMBA, each rat was weekly weighed and subsequently palpated twice weekly throughout 20 weeks period (Figure 3.1).

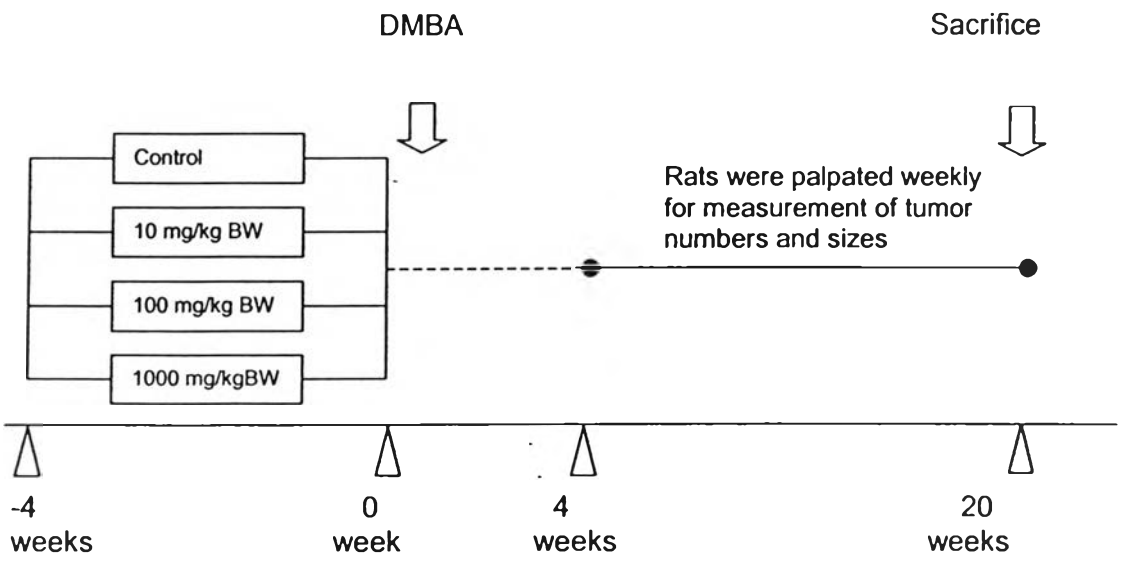

Figure 3.1 Diagram of experiment I

At the end of experiment or at least one palpable mammary tumor exceeded 4 cm in diameter or animals became moribund, the rats were asphyxiated with ether and sacrificed. Liver, ovary and uterus were excised, weighed. The tumors were weighed and measured for volume and subsequently fixed with 10% formalin. Sections (5 μm) for the paraffin-embedded tumors were stained with Haematoxylin and Eosin (H&E) for histological analysis and Estrogens Receptor analysis.

3.5.2 Experiment II: Evaluation of antitumor activities of *P. mirifica* and *B. superba* in DMBA-induced rat mammary carcinoma

One hundred and forty female Spargue-Dawley rats, 50 day-old and 165 to 185 g body weight were selected. The rats were induced for mammary tumor by the single dose treatment of 80 mg/kgBW of 7,12-DMBA. After 7,12-DMBA administrations, rats with developed palpable mammary tumors with approximate diameter of 0.1 cm. were assigned to one of the seven treatments (20 rats per group and 5 rats per cage). The criteria of dividing the rats into each group were examined by the time and location of tumor appearance. Each group was divided into 7 groups and submitted to the same treatment, as did in the experiment I (Figure 3.2).

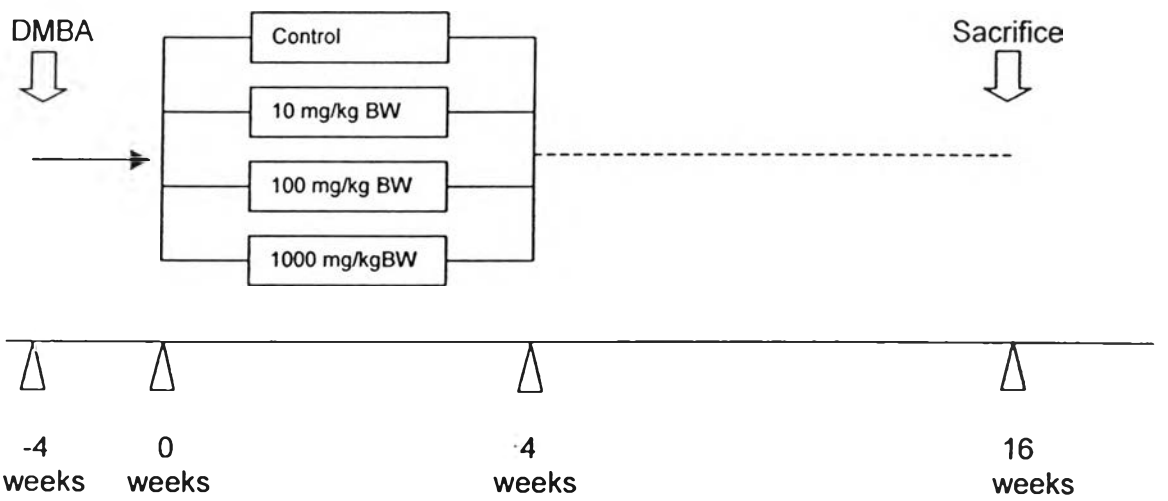

Figure 3.2 Diagram of experiment II

Following the single exposure to 7,12-DMBA, the rat was weekly weighed and subsequently palpated twice weekly throughout 20 weeks period. At the end of

experiment or at least one palpable mammary tumor exceeded 4 cm in diameter or animals became moribund, the rats were asphyxiated with ether and sacrificed. Liver, ovary and uterus were excised, weighed. All tumors were weighed and measured for volume and subsequently fixed in 10% formalin. Sections (5 μm) for the paraffin-embedded tumors were stained with H&E for histological analysis and Estrogens Receptors analysis.

3.6 Evaluation of mammary tumor development

3.6.1 Induction of mammary carcinoma rat

The procedures used were patterned according to the references (Huggins et al. 1959 and 1961). The 50 – 55 day-old rats were gavaged with 80 mg. DMBA/kg BW suspended in corn oil by instillation in the stomach though a soft rubber catheter (0471), 4 cm long and outer diameter 0.1 cm. Following the single exposure to 7,12-DMBA, the rat was weighed and subsequently palpated twice weekly throughout 20 weeks period.

The mammary tumor development was assessed according to the following parameters: the tumor appearance and tumor onset. The number of rats found with detected mammary tumor within 20 weeks after administering with DMBA (Tumor incidence). The mean number of palpable tumor in each treatment group and the tumor location were studied (Figure 3.3 and Table 3.2). Tumor multiplicity or numbers of mammary tumor per rat were recorded. The size of mammary tumors in each animal were measured and followed up weekly.

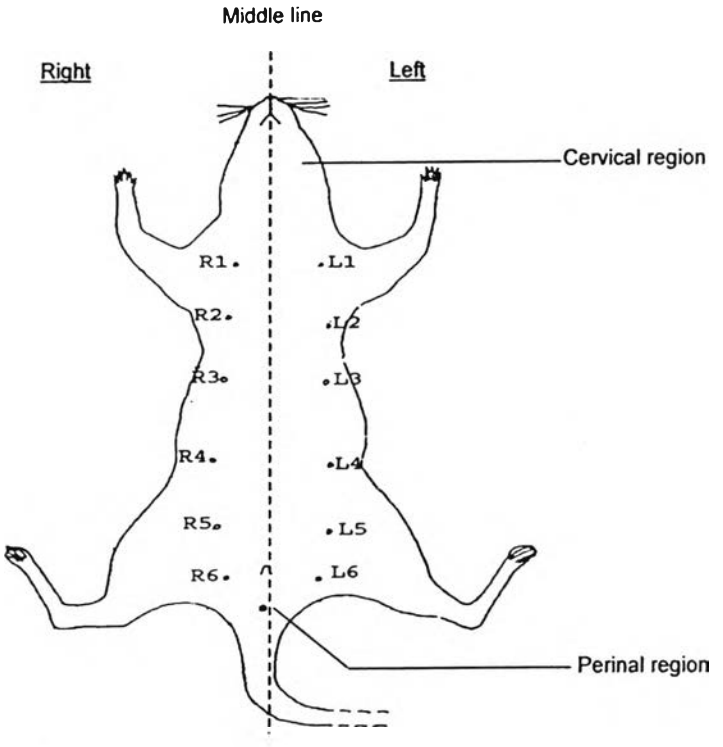

Figure 3.3 Areas of allocation on rats for tumor

Table 3.2 Code for identification of palpable masses

Code	Location	Code	Location
Right side		Left side	
R0	Between cervical region and mammary gland 1	L0	Between cervical region and mammary gland 1
R1	Between mammary gland 1-2	L1	Between mammary gland 1-2
R2	Between mammary gland 2-3	L2	Between mammary gland 2-3
R3	Between mammary gland 3-4	L3	Between mammary gland 3-4
R4	Between mammary gland 4-5	L4	Between mammary gland 4-5
R5	Between mammary gland 5-6	L5	Between mammary gland 5-6
R6	Between mammary gland 6-perinal region	L6	Between mammary gland 6-perinal region

R = Right, L = Left

3.6.2 Tumor pathology

The criteria for identification of the structures are previously described (Young and Hallowes 1973). Mammary tumours were evaluated and classified as follows:

1. Tumor-like lesions
   - Lobular hyperplasia (adenosis)
2. Benign fibroepithelial tumors
   - Adenoma
   - Fibroadenoma
   - Fibroma
3. Malignant epithelial tumors
   - Adenocarcinoma
   - Papillary carcinoma
   - Anaplastic carcinoma
   - Cribriform carcinoma
   - Squamous cell carcinoma
4. Malignant mesenchymal tumors
   - Sarcoma

3.7 Histological study

3.7.1 Chemicals

- Ethyl alcohol
- n-butyl alcohol
- Xylene
- Canada balsam
- Haematoxylin
- Picric acid
- Paraffin
- Ammonia alum
- Glacial acetic acid
- 40% Formaldehyde
- Eosin

3.7.2 Equipments

- Slide
- Cover glass
- Microtome
- Microtome blade
- Hot air oven
- Tissue floating bath
- Light microscope
- pH meter
- Hot plate

3.7.3 Procedures and methods

This study used the standard histological techniques (Humanson, 1979) using Haematoxylin and Eosin staining (Ehrlich, 1886) (see Appendix D) to study the structural feathers of the sections of the liver, ovary, uterus and mammary tumor. The processes were examined 5 steps as follows.

1. Fixation: The tissues were fixed in 10% buffer formalin at least 24 hours after sacrification. The tissues were transferred to newly 10% buffer formalin replacing the turbid one.
2. Dehydration: The tissues were cut into small piece and transferred into ethyl alcohol as follows.

Step 1 90% ethanol	1 time	1 hour/time
Step 2 95% ethanol	2 time	6 hours/time
Step 3 N-butyl alcohol	1 time	1 hour/time
Step 4 Xylene	1 time	1 hour/time

3. Embedding: The tissue was impregnated in the hot air oven (58 °C). The tissue was placed in the embedding compound. One the tissue had been infiltrated; it is placed into a mold and surrounded by wax and allowed to solidify into a block. The step wises were following.

Step 1 Xylene+molten wax (1:1)	1 time ½ hour/time
Step 2 Wax I	1 time ½ hour/time
Step 3 Wax II	1 time 1 hour/time

Step 4 Embedded and orientated in filtered wax.

4. Sectioning: The blocks containing tissue were mounted onto a microtome. The tissue blocks were sectioned at 5 μm. Paraffin sections were mounted onto glass microscopic slides by egg albumin for further processing.
5. Hydration and staining: The glass microscopic slides containing paraffin sections were deparaffined and staining as follows.

1) 2 x 5 minutes	Xylene
2) 1 x 3 minutes	n-butyl alcohol
3) 1 x 3 minutes	95% ethyl alcohol
4) 1 x 3 minutes	70% ethyl alcohol
5) 1 x 3 minutes	tap water
6) 10-12 minutes	Hematoxylin solution
7) 5-10 seconds	Acid alcohol

8) 10 minutes	running tap water
9) 1 x 3 minutes	70% ethyl alcohol
10) 1 x 3 minutes	90% ethyl alcohol
11) 3-5 minutes	Eosin staining solution
12) 15-30 seconds	95% ethyl alcohol
13) 1 x 5 minutes	n-butyl alcohol
14) 1 x 5 minutes	Xylene

Then sides were mounted with cover slip by Canada balsam and laid slides flat while drying.

3.8 Estrogen receptor analysis

Four μm, formalin-fixed paraffin-embedded sections were deparaffinized and rehydrated with through graded concentrations of alcohol to distilled water. To enhance the immunoreaction, the sections were boiled twice in distilled water using a microwave oven for 10 min. Buffer was added in between to prevent the sections from drying. The sections were then washed in phosphate buffer saline (PBS, 0.1M, pH 7.4) and incubated with 3% H_2O_2 in methanol for 10 min. All the procedures after heating were done at room temperature. After another washing in buffer, the sections were pretreated with blocking reagent (normal goat serum in PBS containing carrier protein, Chemicon, USA) for 5 min before incubation with primary antibody for 90 min. The primary antibodies used were rabbit monoclonal antibody to ER-α (PA1-308, Affinity BioReagents, USA) in a dilution of 4 μg/ml and ER-β (PA1-308B, Affinity BioReagents, USA) in a dilution of 1 μg/ml. Negative controls were run by the omission of primary antibody and by the replacement of the primary antibody with PBS.

After primary antibody incubation, the sections were washed in PBS and incubated with biotinylated secondary antibody, for 30 min. Finally, after another washing in PBS, the sections were incubated with avidin biotin complex for 30 min. An immunoreaction was observed by using 3-3' diaminobezindine (DAB kit, Vector) and all sections were counterstained with Meyer's hematoxylin.

Positive cells from control and treated sample were identified in a count from the strongest intensity selected field and a hundred of cells were counted for defining the positive cell. Two independent observers according to a simplified scoring system

determined immunoreactivity: cases were rated negative if none for the cells within the lesions were stained. The percentages of ER-β and ER-α were calculated.

3.9 Quantitative of phytoestrogens in *P. mirifica*, *B. superba*, rat food

The quantitative determination of *P. mirifca* and *B. superba* was based on HPLC-UV methods (Subongkoch, 2002). The determination was also done in rat food and serum by adapted methodology from these reports (Franke et al., 1998). All analyses were performed in duplicate. The method and equipments is following.

3.9.1 Chemical and Reagents

Authentic standards of isoflavonoids including, puerarin, daidzin, genistin, daidzein, genistein and flavone were purchased from Sigma, USA. The organic solvents for chromatography (HPLC grade) were purchased from Merck, Germany. The water of over 16 MΩ/cm for a component of the mobile phase of HPLC was prepared by Maxima Ultrapure Water Systems (ELGA).

β-glucuronidase (EC 3.2.1.3) was isolated from *E. coli* type IX-A and arylsulfatase (EC 3.1.6.1) was isolated from *Helix pomatia* type H-1, which purchased from Sigma-Aldrich, Co. (St. Louis, MO).

3.9.2 Sample collection and preparation

Five gram of *P. mirifica* and *B. superba* tuberous finely ground powders and rat chow diet were extracted twice at room temperature with 50 ml of 70% methanol (Merck) with the aid of sonication for 15 min. The flask was allowed to cool at room temperature and fill to volume with 70% methanol. Methanol extracts were filtered with 0.45 μm pore size, 13 mm diameter membrane into an HPLC vial and cap the vial for further injection. A flow chart of the method is showed in Figure 3.4.

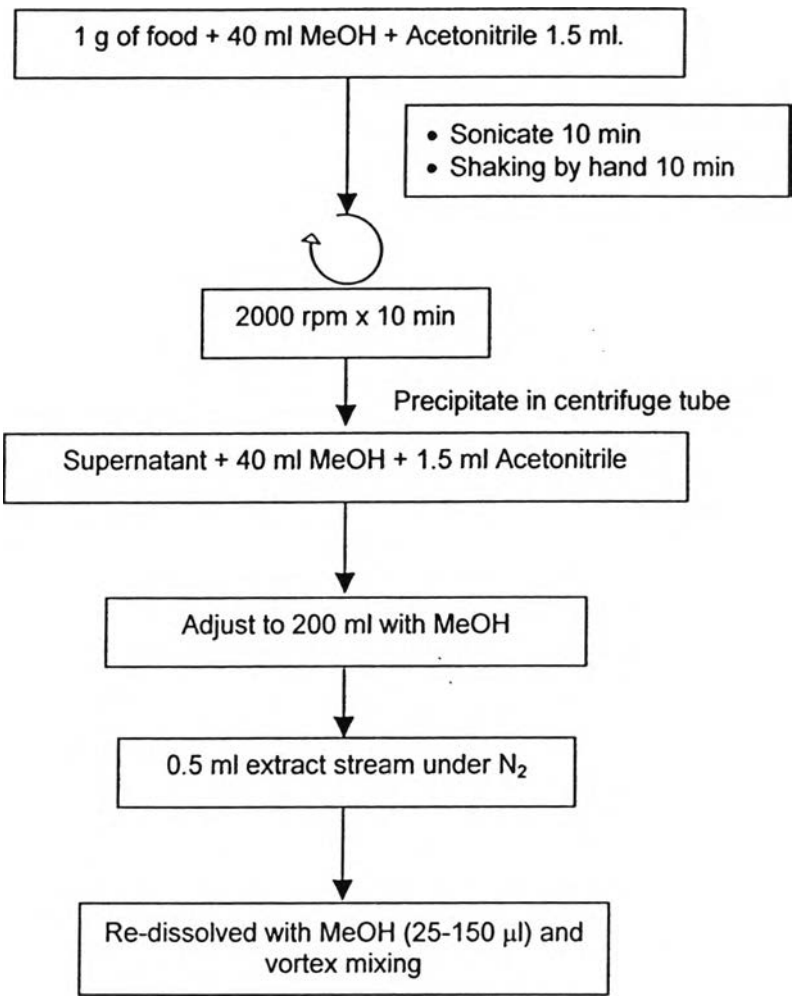

Figure 3.4 Flow chart of the plant and food preparation for HPLC analysis

3.9.3 Standard preparation

The standard isoflavonoids were serially diluted with methanol to establish the concentrations of 1, 0.5, 0.25, 0.01, 0.05, 0.01, 0.005, 0.0025, 0.0010 mg/ml, respectively. A flow chart of the method is showed in Figure 3.5.

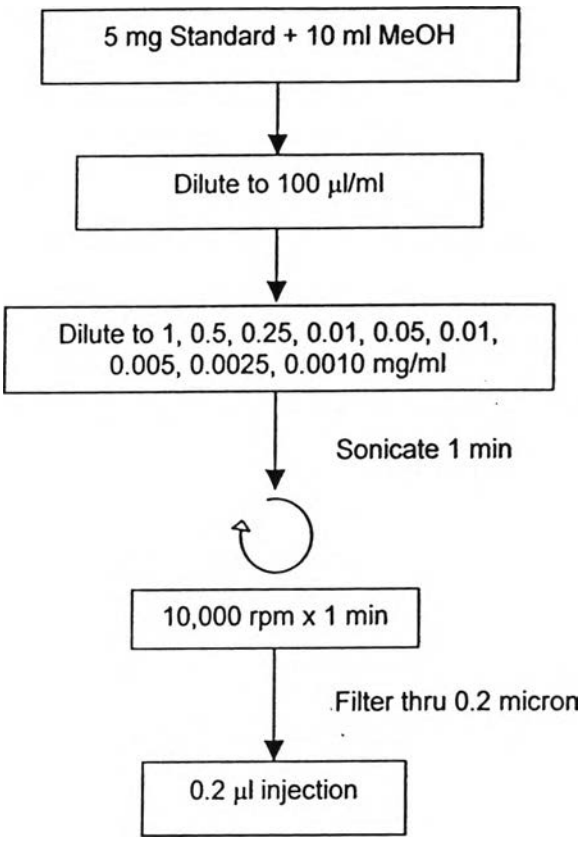

Figure 3.5 A flow chart of the standard sample preparation for HPLC analysis

3.9.4 Apparatus and chromatographic condition

HPLC condition and apparatus was carried as follows.

HPLC system	A Water 600-Rheodyne processing with a Water 717 plus Autosample and Phtotodiode Array detector 2996 (Waters Corp,USA). Chromatography manager was performed by a personal computer using the Empower software
Column	Symmetry C_{18} column (150 x 4.6 nm) was connected with a Sensory guard column (Waters Corp,USA).
Temperature	25 °C
Flow rate	0.8 ml/min.
Injection volume	20 µl.
Running time	40 minutes

Mobile phase Chromatographic system was carried out under gradient system which mobile phase: (A) 0.1% acetic acid and (B) acetonitrile. The gradient solvent system was following.

Retention time (min)	%A	%B
0	86	14
5	86	14
9	76	24
22	68	32
40	86	14

DAD detector Diode-array Detector (DAD) Phtotodiode Array detector 2996 (Waters Corp,USA).

The filter set was Millipore membrane, 0.45 μm pore size with 13 mm diameter for the sample and 47 mm diameter for mobile phase, of HA type for aqueous solution and HV type for organic solvent.

The analysis time was set from the retention time of the last eluted reference standard. The wavelength of the analytical path was 254 nm. Calibration curves were obtained for all isoflavonoids with high linearity by plotting the standard concentration as a function of peak area from HPLC analysis. Ultraviolet detection was done at a wavelength corresponding to the most intense absorption maximum at 254 nm.

3.10 Statistical analysis

The results of all parameters were expressed as the mean ± S.E. Analysis of Variance (ANOVA) was used to determine of 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 $p<0.05$. Except for comparison within tumor development was evaluated by Kruskall-Wallis test followed by the Dunn's test for multiple comparisons. Results from immunohistochemical analysis were evaluated by the Fisher's extract test for proportion.