Chapter 3

Methodology

1. Materials

1.1 Animals

Adult Sprague–Dawley rats of either sex, aged 4 - 5 weeks with a weight of 200 – 250 g were purchased from the National Laboratory Animal Center, Salaya Mahidol University, Nakorn Pathom, Thailand. The rats were kept in an animal room where the temperature is maintained at 25 ± 1°C under a 12 h light–dark cycle. They were provided with a standard diet and water *ad libitum* for one week to be acclimatized before starting the experiment.

1.2 Plant materials

Mature fruits of *T. chebula* were collected during September to December from a joint of Soengsang district, Nakorn Rajsrima province and Prakam district, Buriram province forest. Mature fruits of *T. bellerica* were collected during September to November from the forest of Wangnumyen district, Sakaew province. These plants were identified by Assoc. Prof. Dr. Noppamas Soonthornchareonnon, Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Thailand, where a voucher specimen PBM 00485 (*T. chebula*) and PBM 02678 (*T. bellerica*) have been deposited.

1.3 Chemicals and instruments

The chemicals and instruments employed in the present study were summarized in the table 3.1 and 3.2.

Tale 3.1

List of chemicals used in this study

Name	Source
Formaldehyde solution	Merck (USA)
Sodium phosphate, dibasic (anhydrous)	Merck (USA)
Sodium phosphate, monobasic	Merck (USA)
Ethylenediamine tetraacetic acid di-sodium salt	Merck (USA)
Wright's stain agent	Biotech (Thailand)
Paraffin	Ma Cormick Scientific (USA)
Ethyl alcohol	Merck (USA)
Xylene	Merck (USA)
Hematoxylin	Merck (USA)
Eosin	Merck (USA)
Permouth	Merck (USA)

Table 3.2

List of instruments used in this study

Name	Source
Automated hematology analyzer (SYSMEX SF-300)	Dimond Diagnostics (USA)
Automated analyzer (COBAS INTEGRA SYSTEM)	Roche Diagnostics (USA)
Centrifuge	Hettich (USA)
Tissue embedding	Leica Microtome (USA)
Microtome	RMC Microtome (USA)
Water bath	Bio-optica (Italy)
Light microscope	Olympus Optical Co., (Japan)

2. Methods

2.1 Preparation of plant extract

The dried fruits of *T. chebula* and *T. bellerica* were extracted by the following method, 68 kg of *T. chebula* dried fruits or 104 kg of *T. bellerica* dried fruits were separately boiled with water for 1 hour and filtered, repeated this method 3 times. The water extract of each dried fruit was spray-dried to remove trace of water. After that, the water crude extracts of *T. chebula* and *T. bellerica* were tested for quality control e.g. percentage of loss on drying, total ash, acid insoluble ash, extractive value, microbial test, aflatoxin test, and heavy metal test according to Thai Herbal Pharmacopoeia. Also, these two plants were evaluated for quantity of chemical compounds e.g. percentage of total carbohydrate, percentage uronic acid, monosaccharide and tannin content. Then the dried water crude extract was stored at 4 °C until used.

2.2 Preparation of test substance

Distilled water was used as vehicle for all test substances, at concentrations of 300, 600, and 1,200 mg/ml for chronic toxicity and 1,250 mg/ml for acute toxicity test.

2.3 Test substances administration

All test substances were orally administered in an equivalent volume of 0.1 ml/100 g body weight of the rats. Control groups received vehicle only in the same volume and same route of administration by feeding tube.

2.4 Observation of toxic symptoms

Clinical examination, observation, and mortality check were made shortly after dosing, at frequent intervals over the next 4 hr, and at least once daily thereafter. The interval and frequency of observation were flexible enough to determine the onset of signs, onset of recovery, and the time of death. The most common pharmacotoxic signs that may provide valuable clues to the target organ or system of toxicity of a test substance were listed in table 3.3. Individual body

weight was determined just prior to administer the first dose, once weekly or at death, and at termination.

2.5 Hematological study

2.5.1 Determination of hematological parameters

Blood sample was collected from a common carotid artery and collected into heparinized tube for determining the hematological parameters (RBC count, hematocrit, hemoglobin, MCV, MCH, MCHC, WBC, and platelet count).

2.5.2 Determination of percent white blood cells

Blood sample was collected from a common carotid artery into heparinized tube. The blood was smeared and left for drying. The dried smears were fixed by dipping into absolute methanol for 5 dips and stained using Wright's stain method. Thereafter, blood smear slides were used to determine the percent of white blood cells. Different types of white blood cells (neutrophil, lymphocyte, monocyte, eosinophil, and basophil) were determined by specifying their type in 100 cells.

2.6 Biochemical study

Blood sample was collected from a common carotid artery into non-heparinized tube. It was allowed to coagulate before it was centrifuged at 3,000 rpm for 10 minutes. The serum was evaluated by Cobas integra automatic from Roche Company. The serum was quantitated for glucose, creatinine, BUN, total protein, albumin, total bilirubin, direct bilirubin, AST, ALT, and ALP.

2.7 Pathological study (Carson, 1996; Feldman & John, 1988; Hayes, 2001)

2.7.1 Gross examination

Gross and pathological examinations of the internal organs were performed to confirm whether or not the organs or tissues had been damaged. A gross necropsy was observed any abnormality according to its size, shape, color, lesions, location, severity, and number.

The lesions were used to denote a tissue change arising from any one of a wide variety of disease conditions such as a lesion was produced by an infectious agent, toxicity, neoplasia, inherent genetic or age-related change. The lesions were very characteristic, for instance, hydronephrosis, abscess, laceration, mass, adhesions, cyst, deformity, enlarged, fluid, rupture, fracture, etc. In addition, the lesions were also measured for the number and size in three dimensions. The color changes such as pale, yellow, yellowish-brown, gray, green, black, or white were described as well. Moreover, the changing in shape of the organs or the lesions was also reported. Symmetry was very important in the consideration of tissue alteration. Paired organs, for instance, were compared to one another for detection of subtle changes. The texture or consistency of the organs or the lesion was also revealed. The appearance of the surface of organ or lesion was usually described as homogenous or heterogeneous. Finally, the severity of lesions or any organ change was often reported by terms of minimal, mild, moderate, or marked changes.

2.7.2 Specimen collection and weight

The organs such as lungs, heart, livers, spleen, adrenals, kidneys, pancreas, brain, testes, epididymis, ovaries and uterus were removed, blotted free of blood and weighed immediately on an electronic balance for subsequent analysis. The organs include eyes, thymus, stomach and duodenum, small intestine, muscle with the sciatic nerve and thoracic spines were also collected and observed.

Table 3.3

Common signs and observation in toxicity test

Clinical observation	Observed signs	Organs, tissues, or systems most likely to be involved
1. Respiratory: blockages in the nostrils, changes in rate and depth of breathing, changes in color of body surfaces	A. Dyspnea: difficult or labored breathing, essentially gasping for air, respiratory rate usually slow	
	1. Abdominal breathing: breathing by diaphragm, greater deflection of abdomen upon inspiration	Central nervous system respiratory center, paralysis of costal muscles, cholinergic inhibition
	2. Gasping: deep labored inspiration, accompanied by a wheezing sound	Central nervous system respiratory center, pulmonary edema, secretion accumulation in airways (increase cholinergic)
	B. Apnea: a transient cessation of breathing following a forced respiration	Central nervous system respiratory center, pulmonary-cardiac insufficiency
	C. Cyanosis: bluish appearance of tail, mouth, foot pads	Pulmonary-cardiac insufficiency, pulmonary edema
	D. Tachypnea: quick and usually shallow respiration	Stimulation of respiratory center, pulmonary-cardiac insufficiency
	E. Nostril discharges: red or colorless	Pulmonary edema, hemorrhage

Table 3.3 (Continued)

Clinical observation	Observed signs	Organs, tissues, or systems most likely to be involved
2. Motor activities: changes in frequency and nature of movements	A. Decrease or increase in spontaneous motor activities, curiosity, preening, or locomotion	Somato-motor, central nervous system
	B. Somnolence: animal appears drowsy, but can be aroused by prodding and resumes normal activities	Central nervous system sleep center
	C. Loss of righting reflex: loss of reflex to maintain normal upright posture when placed on the back	Central nervous system, sensory, neuromuscular
	D. Anesthesia: loss of righting reflex and pain response (animal will not respond to tail and toe pinch)	Central nervous system, sensory
	E. Catalepsy: animal tends to remain in any position in which it is placed	Central nervous system, sensory, neuromuscular, autonomic
	F. Ataxia: inability to control and coordinate movement while animal is walking with no spasticity, epraxia, paresis, or rigidity	Central nervous system, sensory, autonomic
	G. Unusual locomotion: spastic, toe walking, pedaling, hopping, and body posture	Central nervous system, sensory, neuromuscular

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Table 3.3 (Continued)

Clinical observation	Observed signs	Organs, tissues, or systems most likely to be involved
2. Motor activities: changes in frequency and nature of movements	H. Prostration: immobile and rests on belly	Central nervous system, sensory, neuromuscular
	I. Tremors: involving trembling-quivering of the limbs or entire body	Neuromuscular, central nervous system
	Fasciculation: involving movements of muscles, seen on the back, shoulders, hind limbs, and digits of the paws	Neuromuscular, central nervous system, autonomic
3. Convulsion (seizure): marked involuntary contraction or seizures of contraction of voluntary muscle	A. Clonic convulsion: convulsive alternating contraction and relaxation of muscles	Central nervous system, respiratory failure, neuromuscular, autonomic
	B. Tonic convulsion: persistent contraction of muscles, attended by rigid extension of hind limbs	Central nervous system, respiratory failure, neuromuscular, autonomic
	C. Tonic-clonic convulsion: both types may appear consecutively	Central nervous system, respiratory failure, neuromuscular, autonomic
	D. Asphyxial convulsion: usually of clonic type, but accompanied by gasping and cyanosis	Central nervous system, respiratory failure, neuromuscular, autonomic

Table 3.3 (Continued)

Clinical observation	Observed signs	Organs, tissues, or systems most likely to be involved
3. Convulsion (seizure): marked involuntary contraction or seizures of contraction of voluntary muscle	E. Opisthotonos: titanic spasm in which the back is arched and the head is pulled towards the dorsal position	Central nervous system, respiratory failure, neuromuscular, autonomic
4. Reflexes	A. Corneal (eyelid closure): touching of the cornea causes eye lids to close	Sensory, neuromuscular
	B. Pinnal: twitch of external ear elicited by light stroking of inside surface of ear	Sensory, neuromuscular, autonomic
	C. Righting	Central nervous system, sensory, neuromuscular
	D. Myotact: ability of animal to retract its hind limb when limb is pulled down over the edge of a surface	Sensory, neuromuscular
	E. Light (pupillatory): constriction of pupil in the presence of light	Sensory, neuromuscular, autonomic
5. Ocular signs	A. Lacrimation: excessive tearing, clear or colored	Autonomic
	B. Miosis: constriction of pupil regardless of the presence or absence of light	Autonomic

Table 3.3 (Continued)

Clinical observation	Observed signs	Organs, tissues, or systems most likely to be involved
5. Ocular signs	C. Mydriasis: dilation of pupils regardless of the presence or absence of light	Autonomic
	D. Exophthalmos: abnormal retraction of eye in orbit	Autonomic
	E. Ptosis: dropping of upper eyelids, not reversed by prodding animal	Autonomic
	F. Chromadacryorrhea (red lacrimation)	Autonomic, hemorrhage, infection
	G. Relaxation of nictitating membrane	Autonomic
	H. Cornea opacity, iritis, conjunctivitis	Irritation of the eye
6. Cardiovascular signs	A. Bradycardia: decreased heart rate	Autonomic, pulmonary-cardiac insufficiency
	B. Tachycardia: increased heart rate	Autonomic, pulmonary-cardiac insufficiency
	C. Vasodilation: redness of skin, tail, tongue, ear, foot pad, conjunctivae sac and warm body	Autonomic, central nervous system, increase cardiac output, hot environment
	D. Vasoconstriction: blanching or whitening of skin, cold body	Autonomic, central nervous system, cold environment, cardiac output decrease

Table 3.3 (Continued)

Clinical observation	Observed signs	Organs, tissues, or systems most likely to be involved
6. Cardiovascular signs	E. Arrhythmia: abnormal cardiac rhythm	Central nervous system, autonomic, cardiac-pulmonary insufficiency, myocardial infarction
7. Salivation	A. Excessive secretion of saliva: hair around mouth becomes wet	Autonomic
8. Piloerection	A. Contraction of erectile tissue of hair follicles resulting in rough hair	Autonomic
9. Analgesia	A. Decrease in reaction to induced pain (e.g. hot plate)	Sensory, central nervous system
10. Muscle tone	A. Hypotonia: generalized decrease in muscle tone	Autonomic
	B. Hypertonia: generalized increase in muscle tone	Autonomic
11. Gastrointestinal signs: dropping (feces)	A. Solid, dried and scant	Autonomic, constipation, Gastrinal intestines motility
	B. Loss of fluid, watery stool	Autonomic, diarrhea, Gastrinal intestines motility
Emesis	A. Vomiting and retching	Sensory, central nervous system, autonomic (in rat, emesis is absent)
Diuresis	A. Red urine (Hematuria)	Damage in kidney
	B. Involuntary urination	Autonomic, sensory

Table 3.3 (Continued)

Clinical observation	Observed signs	Organs, tissues, or systems most likely to be involved
12. Skin	A. Edema: swelling of tissue filled with fluid	Irritation, renal failure, tissue damage, long term immobility
	B. Erythema: redness of skin	Irritation, inflammation, sensitization

(Chan & Hayes, 1989)

2.7.3 Histopathological examination

Histopathology refers to the microscopic examination of tissue in order to study the manifestations of disease. The histopathology laboratory processed formalin fixed tissues and prepared glass slides for microscopic examination of tissues. The tissues were stained with hematoxylin and eosin (H&E). The tissue was observed for any abnormality according to its size, shape, staining, lesions, location, severity, and number. The alterations of a cell or tissue in response to adverse environmental changes refer to cellular adaptation. Five major types of adaptation including atrophy, hypertrophy, hyperplasia, dysplasia, and metaplasia were determined. Atrophy is a decrease in cell size. Tissue and organs especially susceptible to atrophy include skeletal muscle, cardiac muscle, and the brain. Hypertrophy is an increase in cell size. The heart and kidneys have increased susceptibility to hypertrophy. An example of pathologic hypertrophy is in cardiac muscle as a result of hypertension. Hyperplasia is an increase in the number of cells. It is the result of increased cell mitosis, or division. Pathologic hyperplasia is an abnormal increase in cell division. Dysplasia refers generally to abnormal changes in cellular shape, size, and/or organization. Metaplasia occurs when a differentiated cell of a certain type is replaced by another cell type, which may be less differentiated.

The histopathological examination of tissue was determined by microscope. To obtain H&E stained slides, several steps were typically performed after tissue fixation including, dehydration, clearing, and infiltration. Samples were

subsequently developed, comprising of embedding, sectioning, staining, and mounting, respectively.

2.7.3.1 **Fixation**

A fixative agent alters tissue by stabilizing the protein so that fixed tissue was resistant to further changes. The postmortem activities of decay, or putrefaction (bacterial attack), and autolysis (enzyme attack) can be prevented by fixation. Another function of fixatives maintains the proper relationship between cells and extracellular substances such as the connective tissue fibers (collagen, reticulin, and elastin) and amorphous ground substance. Ten percent formalin was used as fixative agent which was the most commonly used and was probably one of the most valuable, even though it was not considered to be the best fixative for subsequent paraffin infiltration. The composition of the fixative agent was shown in table 3.4. The pH of the solution was approximately 6.8 and hypotonic.

Table 3.4

Ingredient of 10 percent neutral buffered formalin

Compound	Volume
Formaldehyde, 37% to 40%	100 ml
Distilled water	900 ml
Sodium phosphate, monobasic ($NaH_2PO_4H_2O$)	4 g
Sodium phosphate, dibasic (Na_2HPO_4)	6.5 g

2.7.3.2 **Processing**

The process after fixation was dehydration, clearing, and infiltration.

2.7.3.2.1 **Dehydration**

It was necessary in the preparation of tissue block for embedding in paraffin medium. The dehydrating reagent was absolute alcohol. The whole process is called dehydration

2.7.3.2.2 Clearing

Clearing was performed at two different occasions, (1) during tissue processing and (2) soon after the specimen staining for microscopic examination. Xylene, the aromatic hydrocarbon, was used as a clearing agent. Specimens were washed with xylene twice.

2.7.3.2.3 Infiltration

After dehydration and clearing, tissue must be infiltrated with the supporting medium. This medium was generally referred to an embedding medium. While thin sections were cut, this embedding medium held together the cells and intercellular structures in their proper shape. Paraffin wax was generally used as embedding medium. A large numbers of tissue blocks were processed in a short time and serial sections were obtained. Then, routine and special staining was done. The protocol for infiltration was shown in table 3.5.

Table 3.5

The protocol for infiltration

Sequent	Solution	Time period
1	95% Ethyl alcohol	overnight
2	95% Ethyl alcohol	30 minutes
3	Absolute ethyl alcohol	30 minutes
4	Absolute ethyl alcohol	30 minutes
5	Xylene	30 minutes
6	Xylene	1 hour
7	Paraffin	2 hours
8	Paraffin	2 hours

2.7.3.3 Embedding

Using the automatic tissue processor for embedding, tissues were soaked in melting paraffin wax, embedded down in the mold covered with melting paraffin, and then covered with a plastic lid. After these embedded tissues were solidified, they were removed from the mold.

2.7.3.4 Sectioning

The paraffin sections of each tissue were cut at 3 microns thickness using a microtome. Slide coated with gelatin was used as section adhesives. After that, all the sectioned tissue slides were incubated in an oven at 60°C for 1 hour to remove paraffin. Then, the slide section was stained with hematoxylin and eosin.

2.7.3.5 Staining

Hematoxylin and Eosin solution were used for staining. The steps of staining were shown in table 3.6.

2.7.3.6 Mounting

A portion of the stained tissue was mounted on a slide and covered with a cover slip. This enables to keep the specimens for a longer period before the microscopic examination.

3. Experimental protocols

3.1 Acute toxicity

According to the WHO guideline (WHO, 2000) and OECD guideline for testing of chemicals (OECD, 2001a), ten rats of either sex were divided into two groups of five rats per sex. Group 1, a control group was received distilled water and group 2, a test group was received a single oral dose of 5,000 mg/kg body weight.

Observations of toxic symptoms were made and recorded systematically at one, two, four and six hours after administration. The number of survival rats was noted after 24 hours and these rats were then maintained for a further 14 days with observations performed daily for clinical signs of toxicity. These included changes in the skin, fur, eyes, mucous membranes, respiratory, and circulatory functions, autonomic and central nervous system, somatomotor activities as well as behavioral pattern. At the end of the experiment, all surviving rats were sacrificed with an injection of pentobarbital. Their internal organs such as heart, lungs, liver, spleen, kidneys, adrenals, pancreas, brain and sex organs were excised and weighed.

Table 3.6

Step of staining for hematoxylin and eosin

Sequent	Step of staining	Time period
1	Xylene, three changes	2 minutes each
2	Absolute alcohol	10 dips
3	Alcohol, 95%, two changes	10 dips
4	Tap water	Rinse until water runs off evenly*
5	Hematoxylin, Harris, with acetic acid	1 to 3 minutes†
6	Tap water, two changes	10 dips each* or running water may be used if convenient
7	Ammonia water, 0.25% or lithium carbonate, 0.5%	until blue≠
8	Tap water, two changes	10 dips each* or running water may be used if convenient
9	Eosin	10 to 20 dips or eosin-phloxine 1 to 3 minutes
10	Alcohol, 95%, two changes	10-15 dips each
11	Absolute alcohol, three changes	10-15 dips each
12	Xyline, three changes	10-15 dips each

Let slides remained in last container until a cover slip was applied.

* Change water frequently; where two changes were indicated, one container should be changed after each basket. Rotate the containers so that the clean water was in the second container.

† With a large volume of slides, it was best to use a staining time of 1 minute with fresh Harris hematoxylin staining solution, and added 30 seconds per day until 3 minutes of staining time was reached, and then changed to fresh solution.

≠ Do not agitate in the ammonia water, as most section loss could occur at this point. This step requires 10 to 30 seconds, and the solution should be changed when it became discolor.

The pathological observations of these tissues were performed on gross and microscopic bases. Histological examinations were determined on the preserved tissues with particular emphasis on those tissues showing gross pathological changes. The toxicological effect was assessed on the basis of mortality, which was expressed as LD_{50}. If a test at one dose level of at least 5,000 mg/kg body weight did not cause detectable mortality, then a full study using three dose levels might not be necessary.

3.2 Chronic toxicity study

The method was carried out following the protocol described by the WHO guideline (WHO, 2000) and the OECD guideline for testing of chemicals (OECD, 1981b). The rats were divided into five groups of ten females and ten males. Group 1 was a control group which was received distilled water and group 2 – 4 were test groups which were treated with three dose levels of 300, 600 and 1,200 mg/kg body weight/day for a period of 270 days, respectively. Group 5 was a satellite group which was administered with 1,200 mg/kg body weight/day for a period of 270 days and kept for another 28 days post treatment in order to observe for reversible of toxicity.

All rats were weighed and observed daily for clinical signs of toxicity, physiological and behavioral responses. Any rats that die during the experimental period were tested pathologically, and all rats were examined at the end of the experimental period. At the end of the treatment period, all surviving rats were fasted overnight and anesthetized afterwards for blood collection from a common carotid artery. Blood samples were collected into heparinized tube for hematological study. The non-heparinized blood was allowed to coagulate before centrifuged and the serum was separated. The serum was assayed for biochemical examination. Immediately after the blood collection, the rats were sacrificed for tissue studies. The organs were also observed and fixed in 10% neutral buffered formalin solution for a pathological examination.

4. Statistical analysis

The data from the experiments were expressed as mean and standard error of mean (SEM.). Numerical representation of the results was shown as mean ± SEM.

Statistical comparison between groups was analyzed by using one-way analysis of variance (ANOVA), post hoc least-significant difference (LSD) and student's t-test. Statistical differences were considered significant if p values were less than 0.05.