Chapter 5

Conclusion and Recommendation

The use of medicinal plants and preparations derived from them as dietary supplements, nutraceuticals, functional foods and herbal medicinal products has become more widely accepted in Thailand. Therefore, it is important to evaluate the adverse effects of these plants and their preparations. Among these, *T. chebula* and *T. bellerica* have received high attention because decoction of their fresh and dried fruits is used in traditional medicine for the treatment of several illnesses such as laxative, carminative, expectorant, astringent and tonic. Moreover, *T. chebula* and *T. bellerica* have been reported to possess antibacterial, antifungal, antioxidant, and anticancer activities, thus being an initiative material for the development of pharmaceutical products in the future. However, for human safety, acute and chronic toxicity on *T. chebula* and *T. bellerica* should be obtained although they have long been used in traditional medicine. Therefore, the aim of this study was to evaluate the acute and chronic toxicities of water extracts from dried fruits of *T. chebula* and *T. bellerica* in both female and male rats.

The acute toxicity study of *T. chebula* and *T. bellerica* aimed to identify adverse effects of a single dose or multiple doses of the water extract given orally to groups of rats within 24 hours. This study also aimed to find target organs of toxicity of these extracts. The data obtained from these studies would determine the dose of *T. chebula* and *T. bellerica* to be tested for subchronic or chronic toxicities. Acute toxicity was measured by clinical observations or signs of toxicity, body weight, organ weight, gross and pathological examinations of internal organs in rats (Carol, 1995; Chengelis, 1995).

In the acute toxicity study, the water extract from either *T. chebula* or *T. bellerica* with the dose of 5,000 mg/kg body weight was administered orally to both sexes of rats. The first indicator to be evaluated was signs of toxicity. Signs of toxicity (clinical observations) are the most important indicator of drug or chemical related toxicity or morbidity in all types of toxicity studies. Clinical signs including behavior, movements, reflexes, changes in the skin and fur, eyes and mucous

membranes, respiratory, circulatory, autonomic, and central nervous system, were observed according to standardized protocol of OECD guideline (Carol, 1995; OECD, 1981a). The results of this study indicated that water extracts from both plants did not cause any morbidity, morality, abnormal respiratory and cardiovascular pattern to both female and male rats similar to those of ethanol extracts previously done by other groups (Mokkhasmit Swatdimongkol, & Satrawaha, 1971; Thanaporn Jaijoy, Thamaree, Ingkaninan, & Panthong, 2006).

The body weight is the second most sensitive indicators of the condition of an animal if it is monitored frequently and carefully during a study. Rapid or marked body weight loss is usually a harbinger of ill health or death. Rapid body weight loss can be due to decreased feed or water consumption, disease, or specific toxic effects from extracts (Hayes, 2001). The results of this study in both female and male rats did not reveal significant differences in body weight.

In addition to those two parameters, gross and pathological examinations of the internal organs were also performed to further confirm whether or not the organs or tissues had been damaged (Hayes, 2001). In this study, all rats were observed in size, shape, color, lesions, and extent of organs. The results showed no macroscopic or microscopic changes in these internal organs or tissues in any treated rats. Similar to these results, Kimbli reported that the water extract from dried fruit of *T. chebula* administered orally to mice and rats at doses ranging from 50 mg to 1 g per kg body weight for 4 weeks did not show deleterious changes in vital organs including liver or renal (Kimbli, 1997).

In conclusion, the results suggest that the water extracts from dried fruit of *T. chebula* and *T. bellerica* are practically not toxic after an acute exposure in rats, which is similar to previous studies. For *T. chebula*, Mokkhasmit et al. (1971) reported that 50% ethanol extract from fruits of *T. chebula* administered to mice orally and subcutaneously at 1,000, 3,000, and 10,000 mg/kg body weight exhibited no toxic effects (Mokkhasmit, Swatdimongkol, & Satrawaha, 1971). In addition, Dahanukar demonstrated that the water extract from dried fruits of *T. chebula* at doses ranging from 50 mg/kg body weight to 10,000 mg/kg body weight, which was administered orally to mice and rats, did not reveal any toxic effects or mortality (Dahanukar, 1986).

For *T. bellerica*, Mokkhasmit et al. (1971) reported that 50% ethanol extract of its fruits given orally to mice at the dose of 10,000 mg/kg body weight did not cause any toxic effects, but the extract given subcutaneously to mice showed toxic effects with LD_{50} of 6,150 mg/kg body weight (Mokkhasmit et al., 1971). In addition, the study of acute and subacute oral administration of the 95% ethanol extract from its fruits in rats at the dose of 5,000 mg/kg body weight and 1,000 mg/kg body weight, respectively, revealed no toxicity (Thanaporn, Jaijoy, Thamaree, Ingkaninan, & Panthong, 2006).

In chronic toxicity study, both female and male rats in each experiment were given with three different doses of the water extract from dried fruits of *T. chebula* and *T. bellerica* (300, 600, and 1,200 mg/kg body weight/day) for 270 days. In the aspect of general behaviors, both male and female rats treated with these three doses presented no signs of behavior changes and toxicity.

The female groups treated with extracts of *T. chebula* and *T. bellerica* revealed slightly significant differences in body weight gain and internal organ weight. The male groups treated with both extracts showed significant decreases in the body weight and body weight gain. However, the result from monitoring animal health in the entire period of 270 days showed no sign of morbidity and diseases. Furthermore, both female and male rats were healthy as shown by the normal appearance of general behavior, respiratory pattern, cardiovascular signs, motor activities, reflexes, and normal change in skin and fur. Consequently, the affected body weight and body weight gain may have resulted from physiological change in rats such as decrease or increase food intake and metabolic state.

Hematological values were also assessed in order to evaluate any toxic effects on function of bone marrow. Bone marrow is one of the target sites for the adverse effects of test substances. Blood cells are mainly produced in bone marrow. Any test substance that affects the bone marrow could inhibit certain enzyme activities involved in the production of hemoglobin in red blood cells, thereby reducing the ability of the blood to distribute oxygen through out the body, a condition known as anemia. In addition, the morphological examination of red blood cells and white blood cells indicate their production or destruction.

Moreover, white blood cell count was also performed in order to evaluate immune system. There are five types of white blood cells namely neutrophil, eosinophil, basophil, lymphocyte, and monocyte. Neutrophils, which deal with defense against bacterial or fungal infection and other very small inflammatory processes and are usually first responders to microbial infection. Eosinophils primarily deal with parasitic infections. Basophils are chiefly responsible for allergic and antigen response by releasing the chemical histamine causing inflammation. Lymphocytes are responsible for immune responses. There are two main types of lymphocytes: B cells and T cells. The B cells make antibodies that attack bacteria and toxins while the T cells attack body cells when they have been taken over by viruses or have become cancerous. Lymphocytes secrete products (lymphokines) that modulate the functional activities of many other types of cells and are often present at site of chronic inflammation. Monocytes have two main functions in the immune system, the first of which is to replenish resident macrophages and dendritic cells under normal states, and the second of which is to respond inflammation signals. Monocytes can move quickly (approx 8-12 hours) to sites of infection in the tissues and divide/differentiate into macrophages and dendritic cells to elicit and immune response (Barry, 1995; Feldman, Zinkl, Jain, & Moor, 2000; Gregg & Voigt, 2000; Williams, Nelson, & Morris, 1990).

In this study, most values in the treated groups were normal in comparison with the control group. There were some values and some doses that were significantly different from those of the control group such as red blood cell, hematocrit, MCV, MCH, MCHC, neutrophil, lymphocyte, monocyte, and eosinophil. However, such values were within the normal ranges. These variations may have resulted from normal variation among animal groups (Feldman et al., 2000; Inala et al., 2002). Therefore, these results suggest that both *T. chebula* and *T. bellerica* extracts do not cause hematological or immunological defects.

In addition, biochemical examination was performed in order to evaluate any toxic effects on liver, kidney, and glucose metabolism. The parameters such as AST, ALT, ALP, total protein, albumin, total bilirubin, direct bilirubin, BUN, creatinine, and glucose level were examined.

With regard to liver enzyme, AST is normally abundant in heart and liver tissue and is moderately present in skeletal muscle, kidney, and pancreas. In cases of acute cellular injury to the heart or liver, the enzyme is released into the blood from damaged cells and is presumably metabolized within the body. In clinical practice, AST determinations are used to evaluate myocardial injury and to diagnose and assess the prognosis of liver disease resulting from hepatocellular injury (Levine, 1995; Young & Holland, 1995; Sacher & McPherson, 1991).

In this study, most AST levels in groups each treated with *T. chebula* or *T. bellerica* extracts were normal in comparison with the control group. There were significant differences in AST levels compared to the control group except in female rats treated with 600 mg/kg body weight of *T. chebula* and female rats in satellite group administered with *T. chebula* or *T. bellerica* extracts. However, such differences remained within the normal range (Angkhasirisap et al., 2002; Barry, 1995; Caisey & King, 1980).

ALT was another parameter monitored. This enzyme is essentially found in the same tissues that have high concentrations of AST. In liver diseases, ALT elevations parallel those of AST, although slightly more acute hepatocellular parenchymal damage must occur to produce abnormal values. The ALT is relatively more abundant in hepatic tissue versus cardiac tissue than AST. Although serum concentrations of both AST and ALT increase whenever disease processes affect liver cell structure, the ALT is a more liver-specific enzyme (Levine, 1995; Young & Holland, 1995; Sacher & McPherson, 1991). In this study, the level of ALT in all treated groups showed no significant difference from the control group.

ALP is an enzyme found in all body tissues, with particularly high amounts in the liver, bile ducts, and bone. ALP test is done to diagnose liver or bone disease. The higher than normal ALP levels may be due to bone disease, hepatitis, liver disease, and anemia. Moreover, its low level below normal (hypophosphatasemia) may be due to hepatitis, cirrhosis, and protein deficiency (Caisey and King, 1980; Sacher & McPherson, 1991). In this examination, all ALP levels in treated groups with *T. chebula* or *T. bellerica* extracts were normal in comparison with the control group. There were significant decreases in ALP levels compared with the control group except in male rats treated with *T. chebula* extract in

satellite group. Moreover, female rats treated with *T. bellerica* in satellite group showed significant increase as compared to the control group, but these changes were within the normal range (Angkhasirisap et al., 2002; Barry, 1995).

Total protein levels primarily reflect protein synthesis capacity of the liver. Protein loss resulting from renal disease also induces the synthesis of total protein. Dietary insufficiency, maldigestion, or malabsorption can cause severely depleted level of serum proteins, primarily albumin. Severe liver diseases also decrease serum protein levels. Moreover, renal disease such as glomerular nephritis, the nephrotic syndrome, and severe proximal tubular disease, can result in severe chronic loss of protein. Serum protein levels are very insensitive but specific indicators of protein deficiencies caused by malnutrition or liver disease. Increase in serum total protein can be seen in dehydration and an increase in immunoglobulin is often seen in proteins with monoclonal gammopathies (Koller & Kaplan, 1987; Levine, 1995; Sacher & McPherson, 1991). In all groups of treated rats, all doses of *T. chebula* and *T. bellerica* extracts used did not significantly alter total protein levels. Compared to the control group, almost all treated groups showed significant increase in serum protein levels except in male rats treated with *T. chebula* (600, 1,200 mg/kg body weight) or with *T. bellerica* extract (1,200 mg/kg body weight). However, such changes remained within the normal range (Angkhasirisap et al., 2002; Barry, 1995; Caisey & King, 1980). These data suggest that *T. chebula* and *T. bellerica* extracts do not affect protein metabolism.

Albumin is the protein of the highest concentration in plasma. Albumin transports many small molecules in the blood, for example, bilirubin, calcium, progesterone, and drugs. It is also important in keeping the fluid from leaking out into the tissues. Unlike small molecules such as sodium and chloride, this is because the concentration of albumin in the blood is much greater than it is in the fluid outside of it. Because albumin is made by the liver, decreased serum albumin may result from liver disease. It can also result from kidney disease, which allows albumin to escape into the urine. Decreased albumin may also be explained by malnutrition or a low protein diet (Levine, 1995; Young & Holland, 1995; Sacher & McPherson, 1991). In this examination, all satellites groups of *T. chebula* or *T. bellerica* extracts presented significant decrease in albumin level as compared to the control group.

Bilirubin is primarily a breakdown product of hemoglobin and is formed in the reticuloendothelial system. It is then transferred into the blood where it is almost completely bound to serum albumin. When the bilirubin arrives at the sinusoidal surface of the liver cells, the free fraction is rapidly taken up into the cell and converted primarily to bilirubin diglucuronide. A monoglucuronide is also formed and is predominantly metabolized diglucuronide. The conjugated bilirubin diglucuronide is then excreted into the bile and appears in the intestine where bacteria convert the majority of it to urobilinogen. Most of the urobilinogen is destroyed or excreted in the feces, but some was reabsorbed into the blood. This small amount of urobilinogen in the blood is then taken up into the liver and subsequently excreted along with bile; the other protein is excreted into the urine. The mechanism by which conjugated bilirubin in the liver cell is transferred to the blood is not well understood. However, in many type of liver disease, the conjugated form of bilirubin is increased in blood (Levine, 1995; Young & Holland, 1995). In this study, all satellites groups of *T. chebula* or *T. bellerica* extracts significantly decrease both conjugated and unconjugated bilirubin levels as compared to the control group, but such decreases remained within the normal range.

Besides hepatic, kidney was another importance target organ studied. BUN and creatinine were examined for evaluation of kidney function. BUN is an end product of protein metabolism, which is produced solely by the liver; is transported in the blood; and is excreted by the kidneys. The concentration of BUN reflects renal function because the urea nitrogen in blood is completely filtered at the glomerulus of the kidney, and then reabsorbed and tubularly secreted within nephrons. Acute or chronic renal failure is the most common cause of an elevated BUN. Although the BUN is an excellent screening test for renal dysfunction, it is not sufficiently selective for quantifying the extent of renal disease. A number of factors other than renal function can affect the BUN concentration (Levine, 1995; Young & Holland, 1995). In this study, satellite groups of female treated with *T. chebula* extract and of male rats treated with *T. bellerica* extract demonstrated significant decreases in BUN levels compared with the control group, but such decreases remained within the normal range.

Creatinine is derived from creatine and phosphocreatine, a major constituent of muscle. The formation rate for a given individual is remarkably constant and is primarily determined by an individual's muscle mass or lean body weight. Therefore, the serum creatinine concentration is slightly high in muscle subjects, but unlike the BUN, it is less affected by exogenous factors. Once creatinine is released from muscle into plasma, it is excreted really almost exclusively by glomerular filtration. A decrease in the glomerular filtration rate would result in an increase in the serum creatinine concentration (Levine, 1995; Young & Holland, 1995). In this study, administration of *T. chebula* and *T. bellerica* extracts to all groups of rats did not exhibit significant difference in creatinine levels.

With regard to the metabolic effect, serum glucose was monitored to illustrate the effect on glucose metabolism. The glucose concentration in extracellular fluid is regulated closely by homeostatic mechanism to provide body tissue with a ready source of energy. The plasma glucose concentration is usually measured in either the fasting or postprandial state depending upon the type of information desired. Generally, normal glucose values refer to the plasma glucose concentration in the fasting state (Levine, 1995; Young & Holland, 1995). In this study, administration of *T. chebula* and *T. bellerica* extracts to all groups of rats did not present significant difference in glucose levels, suggesting that *T. chebula* and *T. bellerica* extracts do not affect glucose metabolism.

In this study, significant decreases and increases in biochemical values were also observed in both female and male rats as compared to the control groups. However, these biochemical values were minor changes and remained within the normal range (Angkhasirisap et al, 2002; Barry, 1995; Caisey & King, 1980). These data suggest that *T. chebula* and *T. bellerica* extracts do not affect glucose metabolism, liver and renal functions.

Furthermore, necropsy and histopathological examinations were performed to further confirm whether or not the organs such as liver, kidney, heart, lung, brain, spleen, adrenal, and sex organ had been damaged. For necropsy study, abnormalities of tissues and organs were not detected. The general appearances and the internal organs showed normal structure, size, weight, shape, color, and texture.

Firstly, the liver showed a homogenous soft tissue and pinkish-brown organ with multiple lobes. There were no such changes as pale, cyst, and hard mass in the study. Besides, the lesions of liver which point toward inflammation, congestion, hemorrhage, abscess, and infarction were not found. Also, neither small nor enlarge in size of entire liver was found.

Next, the kidney was normal bean-shaped and red-brown in color and has concave and convex surfaces. The concave surface and the renal hilum is the point at which the renal artery enters the organ as well as the renal vein and ureter leaves the organ. The kidney is surrounded by tough fibrous tissue and the renal capsule is surrounded by perinephric fat, renal fascia, and paranephric fat. In the study, kidneys showed homogenous soft entire organs. Similar to the liver, it did not show some lesions such as hydronephosis, mass, cyst, calculi, infarction, hemorrhage, or congestion.

Heart is located in thoracic cavity and the epicardial surface is smooth and glistening. The amount of epicardial fat, which is surrounding to cushion and protect the heart, is usual. The left anterior descending coronary artery extends down from the aortic root to the apex. From the results, the whole heart did not exhibit diminished or enlarged in size, including some lesions such as neoplasia, myocardial infraction, or pericaditis.

Also, the right and left lungs are covered with parietal and visceral pleura. The right lung has four small lobes and is positioned lateral and dorsi-lateral to the right side of the heart, each of which extends caudally to the heart. The left lung has one lobe and is positioned lateral and dorsi-lateral to the left side of the heart. In this study, the rat lungs showed neither deflation nor hyperinflation, including some lesions such as neoplasia, pneumonia, pleuritis, and granulomatous diseases.

Furthermore, the rat brain did not change in size and shape and had intact components without malformation, atrophy, and lesions that reveal infarction, hemorrhage, edema, hernia, and mass.

In the female rats, the sex organs include the uterus, ovaries, oviducts and vagina, all of which presented normal structure, size, weight, shape, color, and texture. The uterus was grayish and did not show the thickened and spongy appearing

myometrial wall. The ovaries had no changes such as hemorrhage, cyst, hard mass, neoplasia, tumor, and cyst. In the male rats, the sex organs include the testis, epididymis, and penis. The testes are two small oval shaped organs which are grayish-white, of soft elastic consistency and covered by a smooth and transparent membrane. The epididymis showed normal structure, size, weight, shape, color, and texture. The study showed that sex organs of both female and male rats did not contain abscess, hemorrhage, cyst, hard mass, neoplasia, tumor, and cyst. For other internal organs such as spleen, thymus, stomach, intestine, pancreas, and adrenal, these organs had normal structure, size, weight, shape, color, and texture. All together, the necropsy results indicated that all of the internal organs were not damaged, suggesting that *T. chebula* and *T. bellerica* do not cause acute and chronic toxicities to rats.

For histopathological study, abnormalities of the tissue regarding size, shape, staining, lesions, severity, and cellular adaptation were not revealed. There is no histological appearance of fatty change in the liver. Generally, lipid accumulates in vacuole of hepatocyte and show a clear appearance with H&E staining. The most common causes of fatty change are diabetes mellitus, obesity, and severe gastrointestinal malabsorption, some drugs, and toxins such as alflatoxin, carbon tratachoride, etc. In addition, the cirrhosis, which appears as the regenerative nodules of hepatocytes surrounded by fibrous connective tissue bridging between portal tracts, was not detected in this study.

Next, the kidneys were normal in size and shape and their histology disclosed normal staining of the cells. Similarly, the glomeruli had normal sizes and shapes. Epithelium and endothelium of renal corpuscle were intact. Cellular inflammation such as glomerulonephitis, edema, and vacuolation were not noticed in the tubular cells. The renal tubules and interstitium were not refilled with inflammatory cells such as neutrophils and eosinophils. In general, many neutrophils are seen in the tubules and interstitium in a case of acute pyelonephritis. The epithelium containing the ragged tubules is identified due to acute tubular necrosis from ischemia. However, microscopic examination of kidneys in this study did not reveal acute renal infarction, dydronephosis illustrated by pelvis dilatation, renal cell carcinoma, and lipoma.

Microscopic examination of the lungs did not display any hemorrhage and edema. Their histology stained with H&E presented normal airways and blood vessels. No inflammatory cells such as mononuclear and polymorphonuclear, interstitial inflammation, and fibrosis were noticed. Alveoli were not filled with a neutrophilic exudate corresponding to the areas of consolidation seen grossly with the bronchopneumonia. In addition, the loss of alveolar walls with emphysema, a silicotic nodule, and pulmonary thromboembolus were not seen. The hyaline membranes lining alveoli and the nuclei were not hyperchromatic and angular.

Heart histology stained by H&E, presented normal coronary artery, large lumen without any atheromatous plaque, normal proportions of muscular arterial wall, and normal appearance of myocardial fibers in longitudinal section. Also, there was no pink to red recent thrombosis in narrowed coronary. In fact this coronary artery narrowing is due to build up of atherosclerotic plaque, which can lead to angina, ischemia, and infarction. The study revealed intact and clearly visible cross striations and nuclei of the myocardial fibers without hemorrhage, myocardial fiber necrosis, and hypertrophic and hyperplasic myocardial fibers. There was no inflammatory cell infiltration, endocarditis, myocarditas, and pericarditis.

Brain was normal and had no hemorrhage, infarction, necrosis, inflammation, and neoplasm. Histological study of female sex organs including ovary and uterus did not show inflammation, lymphocyte, and hemorrhage.

The cervical histology revealed normal non-keratinizing squamous epithelium maturing from basal layer to surface. Also the vulva epithelium showed normal thickness and arrangement as well as the endometrium showed normal structure, arrangement, and proliferation. The uterus did not contain any abnormalities relating to hyperplasia. The microscopic appearance of ovary appeared normal without a hemorrhagic corpus lutea and hyperplasia. For histological study of male sex organs including testis and epididymis, such organs were normal in size and shape and their histology disclosed normal staining of the cells. In the testis, the seminiferous tubules showed numerous germ cells inconspicuous sertoli cells, and small dark oblong spermatozoa in their center.

Finally, other internal organs including spleen, thymus, stomach, intestine, pancreas, and adrenal had normal size, shape, structure, and component. All together,

the results from histopathological study suggest that all of the internal organs are not injured, thus pointing out that *T. chebula* and *T. bellerica* do not cause any acute and chronic toxicities to rats.

In conclusion, the water extracts from the dried fruits of *T. chebula* and *T. bellerica* given orally to female and male rats did not produce both acute and chronic toxicities. Therefore, these data confirm that these water extracts are safe for the rodent's use. Further study regarding toxicology of these extracts should be carried out in non-rodent or human in order to increase the confidence in their safety for the development of pharmaceutical products in the future.