Chapter 2

Review of Literature

1. *Terminalia chebula* Retz.

Terminalia chebula Retz. is a plant belonging to the family Combretaceae. The common names are Sa-Maw-Thai, Sa-Maw-Abhaya (Central), Maa-na (North), Maa-nae (Karen-Chiang Mai), Maak-nae (Karen-Mae Hong Son), Chebulic Myrobalans, and Myrobalan Wood (MOPH, 2000; Smitinand, 2001).

1.1 Botanical description

T. chebula is a medium-sized or large tree, attaining a height of up to 30 meter, with widely spreading branches and a broad roundish crown. The leaves are elliptic oblong with an acute tip and are cordate at the base and margins entire. The leaves are glabrous with a yellowish pubescence below. The flowers are monoecious, dull white to yellow. They are terminal spikes or short panicles and have a strong unpleasant odor. The fruits are usually smooth or frequently 5-ridged, ellipsoid to ovoid drupes, and yellow to orange brown in color, (MOPH, 2000; Smitinand, 2001) as shown in figure 2.1.

1.2 Phytochemical studies

T. chebula contains tannins which are gallic acid, (Kaur, Grover, Singh, & Kaur, 1998; Naik, Priyadarsini, Naik, Gangabhagirathi, & Mohan, 2004; Saleem, Husheem, Harkonen, & Pihlaja, 2002), chebulic acid, punicalagin (MOPH, 2000), chebulanin, corilagin, neochebulinic acid, ellagic acid, chebulegic acid, chebulinic acid, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyanose, 1,6,-di-O-galloyl-D-glucose, casuarinin, 3,4,6-tri-O-galloyl-D- glucose, terchebulin (MOPH, 2000; Cheng, Lin, Yu, Yang, & Lin, 2003; Jung, Sheu, & Lin, 2004; Lee, Ryu, Choi, Lee, & Ahn, 1995). Besides, fructose, amino acids, succinic acid, betasitosterol, resin, and purgative principle of anthroquinone and sennoside nature are also present (Creencia et al., 1966). It also contains flavonol glycosides, triterpenoids (Saleem, Husheem,

Harkonen, & Pihlaja, 2002) β-sitosterol, saponins, and fixed oil containing particularly ester of palmitic, oleic, and linoleic acids (MOPH, 2000).

Figure 2.1

Terminalia chebula Retz.

1.3 Pharmacological studies

1.3.1 Antibacterial activity

Crude extracts from fruits of *T. chebula* showed antibacterial activity against both gram positive and gram negative bacteria. For example, the alcohol and water extracts showed antibacterial activity against *Helicobacter pylori* on disk diffusion method and the water extract showed the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of 125 and 150 mg/ml, respectively (Malekzadeh, Ehsanifar, Shahamat, Levin, & Colwell, 2001). In addition, the alcohol, methanol and water extracts exhibited antibacterial activity against *Bacillus subtilis (B. subtilis*), *Proteus vulgaris (P. vulgaris), Samonella typhimurium (S. typhimurium), Pseudomonas aeruginosa (P. aeruginosa), Esherichia*

coli (E. coli), and *Staphylococcus aureus* (*S. aureus*) on disk diffusion test (Ahmad, Mehmood, & Mohammad, 1998; Bonjar, 2004a; Kim et al., 2006; Phadke & Kulkarni, 1989; Rani & Khullar, 2004; Sato et al., 1997). The water extract from dried fruits of *T. chebula* strongly inhibited the growth, sucrose induced adherence, and glucan induced aggregation of *Streptococcus mutans (S. mutans)*. Mouthrinsing with 10% solution of the extract inhibited the salivary bacterial count and salivary glycolysis (Jagtap & Karkera, 1999). The isolated gallic acid and its ethyl ester isolated from 50% ethanol extract of *T. chebula* are identified as potent antimicrobial substances against even methicillin-resistant strains of *S. aureus* on disk diffusion test (Sato et al., 1997).

1.3.2 Antifungal activity

The water extract from fruits of *T. chebula* exhibited antifungal activity against a number of pathogenic yeast *Candida albicans* and dermatophytes *Epidermophyton*, *Floccosum, Microsporum gypseum* and *Trichophyton rubrum* (Dutta, Rahman, & Das, 1998; Vonshak et al., 2003). The methanol extract from seed of *T. chebula* showed antifungal activity against Clotrimazole-resistant *Candida albicans* on agar well- diffusion test (Bonjar, 2004b).

1.3.3 Antiviral activity

The water extract from fruits of *T. chebula* showed anti-herpes simplex virus (HSV) activity. It inhibited replication of human cytomegalovirus (CMV) and murine cytomegalovirus (MCMV) *in vitro* and *in vivo* (Yukawa et al., 1996). The water and methanol extracts from dried fruits exhibited moderate inhibitory activities (47.3%-64.1%) on human immunodeficiency virus (HIV) type 1 protease by using high performance liquid chromatography (HPLC) to determine enzyme activity (Kusumoto et al., 1995). Furthermore, the both water and methanol extracts from dried fruits of *T. chebula* showed significant inhibitory activity against HIV type 1 reverse transcriptase with $IC_{50} \leq 50$ μg/ml (el-Mekkawy et al., 1995).

1.3.4 Antioxidant activity

The water extract from fruits of *T. chebula* inhibited γ-radiation induced lipid peroxidation in rats liver microsomes and damage to superoxide dismutase enzyme in rat liver mitochondria. The extract inhibited xanthine/xanthine oxidase activity. It can be concluded that the water extract of *T. chebula* is an excellent scavenger of DPPH radicals and also acts as a potent antioxidant and radioprotector (Naik, Priyadarsini, Naik, Gangabhagirathi, & Mohan, 2004). Moreover, it showed inhibition in the thiobarbituric acid reactive substances (TBARS) formation and restored antioxidant enzyme superoxide dismutase (SOD) from the radiation induced damage and should be also evaluated in terms of ascorbate equivalents by different methods such as cyclic voltammetry, decay of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical by pulse radiolysis and decreased in absorbance of DPPH radicals (Naik et al., 2003). It showed protective effects on the tert-butyl hydroperoxide (t-BuOOH)-induced oxidative injury observed in cultured rat primary hepatocytes and rat liver (Lee, Jung, Yun, & Lee, 2007; Lee et al., 2005). The 75% methanol extract from fruits of *T. chebula* inhibited lipid peroxidation (LPO) and scavenged hydroxyl and superoxide radicals (Sabu & Kuttan, 2002). The ethanol extract from fruits of *T. chebula* inhibited oxidative stress induced by UVB (Na et al., 2004). Six extracts (methanol, chloroform, ethanol, *n*-butanol, organic aqueous, water extract) and four compounds (casuarinin, chebulanin, chebulinic acid, 1,6-di-O-galloyl-β-D-glucose) of *T. chebula* fruits exhibited antioxidant activity at different magnitudes of potency (Cheng, Lin, Yu, Yang, & Lin, 2003). The gallic acid, a component of *T. chebula* showed an effect against ferric nitrilotriacetic acid (Fe-NTA)-induced lipid peroxidation (LPO) and showed restoration of antioxidant enzymes in kidney of male Wistar rats (Prasad, Husain Khan, Jahangir, & Sultana, 2006).

1.3.5 Antidiabetic activity

The 75% methanol extract from fruits of *T. chebula* reduced the blood sugar level in alloxan diabetic rats (Sabu & Kuttan, 2002; Kumar, Arulselvan, Kumar, & Subramania, 2006). The chloroform extract from seeds of *T. chebula* exhibited antidiabetic and retinoprotective activity in streptozotocin induced diabetic

rats (Rao & Nammi, 2006).

1.3.6 Antiproliferation activity

The 70% methanol extract from fruit of *T. chebula*, chebulinic acid and ellagic acid decreased cell viability, inhibited cell proliferation and induced cell death in a dose dependent manner on all cell lines studied such as human (MCF-7) and mouse (S115) breast cancer cell line, human osteosarcoma cell line (HOS-1), human prostate cancer cell line (PC3), a non-tumorigenic and immortalized human prostate cancer cell line (PNTIA). It induced apoptosis at lower concentrations, but at higher concentrations, the major mechanism of cell death is necrosis and growth inhibitory manner (Saleem, Husheem, Harkonen, & Pihlaja, 2002).

1.3.7 Antimutagenic activity

The water extract from fruits of *T. chebula* inhibited γ-radiation induced strand breaks formation in plasmid pBR322 DNA (Naik, Priyadarsini, Naik, Gangabhagirathi, & Mohan, 2004). The acetone extract from fruits exhibited antimutagenic activities by Ames *Salmonella* histidine reversion assay (Arora, Kaur, & Kaur, 2003). A tannin fraction from the dried fruit pulp of *T. chebula* obtained by successfully extracting with 95% ethyl alcohol and ethyl acetate showed effective against S9-dependent mutagen, 2-aminofluorene, 4-nitro-o-phenylenediamine. Nevertheless, it is not effective against 4-nitroquinoline-N-oxide (Kaur, Grover, Singh, & Kaur, 1998).

1.4 Toxicological studies

1.4.1 Cytotoxicity

The ethanol extract from the fruit of *T. chebula* exhibited significant inhibitory activity on oxidative stress and the age-dependent shortening of the telomeric DNA length. In the peroxidation model using tert-butyl hydroperoxide (t-BuOOH), the *T. chebula* extract showed a notable cytoprotective effect on the HEK-N/F cells with 60.5% at a concentration of 50 μg/ml. In addition, the *T. chebula* extract exhibited a significant cytoprotective effect against UVB-induced oxidative

damage. The life-span of the HEK-N/F cells is elongated by 40% as a result of the continuous administration of 3 μg/ml of the *T. chebula* extract compared to that of the control. These observations are attributed to the inhibitory effect of the *T. chebula* extract on the age-dependent shortening of the telomere, length as shown by the Southern blots of the terminal restriction fragments (TRFs) of DNA extracted from subculture passages (Na et al., 2004).

Gallic acid and chebulagic acid isolated from the ethanol extract of *T. chebula* fruits as active principles that blocked the cytotoxic T lymphocyte (CTL)-mediated cytotoxicity. Gallic acid and chebulagic acid inhibited the killing activity of $CD8^+$ CTL clone at IC_{50} values of 30 μM and 50 μM, respectively. Granule exocytosis in response to anti-CD3 stimulation is also blocked by Gallic acid and chebulagic acid at the equivalent concentrations (Hamada et al., 1997). The 90% methanol extract from fruits of *T. chebula* inhibited the melanin production of mouse B16 melanoma cell by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Jin, Li, Ahn, Row, & Kim, 2006).

1.4.2 Hepatoprotective activity

The powder of *T. chebula* fruit mixed with rats chow powder (approximately 5 g/rat/day) by free feeding for 10-14 days caused brown pigmentation of tail and limbs, hepatic lesions including early centrilobular vein disruption (9/13), numbers in parentheses refer to proportion of rats which showed changes, centrilobular sinusoidal congestion (13/13) and marked renal lesions including marked tubular degeneration (11/12), tubular casts, and intertubular congestion in rats. Moreover, the rat liver after 13 days of feeding with 25% seeds of *T. chebula* showed perivenous edema and unidentifiable central vein (Arseculeratne, Gunatilaka, & Panabokke, 1985). In addition, the ethanol extract from the fruits of *T. chebula*, which is chemically characterized on the basis of chebuloside II as a marker, is investigated for hepatoprotective activity against anti-tuberculosis drug-induced toxicity. Researchers have found that *T. chebula* extract significantly prevented the hepatotoxicity caused by the administration of rifampicin, isoniazid and pyrazinamide (in combination) in a sub-chronic mode (12 weeks). The hepatoprotective effect of *T. chebula* extract could be attributed to its prominent anti-oxidative and membrane

stabilizing activities. The changes in biochemical observations are supported by histological profile (Tasduq et al., 2006).

1.4.3 Acute toxicity

The acute toxicity study of the 50% alcoholic extract from fruits of *T. chebula* demonstrated no toxic effects for instance behavior, convulsion, and mortality with the dose of 10 g/kg BW when administrated subcutaneously and orally to mice. The amounts of *T. chebula* which are recommended for the traditional usage are 0.04, 0.1, 0.15, 0.35, and 0.5 g/kg (Mokkhasmit, Swatdimongkol, & Satrawaha, 1971). Furthermore, the water extract of the dried fruits of *T. chebula* was administered orally to two different animal species, mice and rats, in doses ranging from 50 mg/kg to 1 g/kg. The extract did not exhibit any toxic effects or mortality throughout the period of the observation at any of the tested dose levels (Dahanukar, 1986).

1.4.4 Subacute toxicity

The water extract from dried fruits of *T. chebula* was administered orally to two different animal species, mice and rats, at various doses ranging from 50 mg/kg to 1 g/kg. The treatment duration was maintained for 4 weeks. No deleterious changes were detected in vital organ function tests including liver or renal function parameters (Kimbli, 1997).

1.4.5 Subchronic toxicity

The subchronic toxic effects of *T. chebula* were studied in which mice were orally administered with a powder of dried fruits at doses of 0.5, 2.5, and 5.0 g/kg body weight/day, 5 days per week for 13 weeks. In a separate study, the other groups of mice were orally administered a water extract of dried fruits at doses of 0.2, 1.0, and 5.0 g/kg body weight, daily for 13 weeks. Both studies showed no adverse effects on body weight, blood biochemistry (blood glucose level, plasma glutamic oxaloacetate transminase and plasma glutamic pyruvic transaminase activities, and blood urea nitrogen level), hematology parameter (total red blood cell count, hematocrit, hemoglobin, mean corpuscular volume, total white blood cell count,

differential leukocyte count; lymphocytes, monocytes, granulocytes), relative organ weight, and histology of liver, kidney, spleen, and thymus (Worasuttayangkurn, 2001).

2. *Terminalia bellerica* (Gaertn.) Roxb.

Terminalia bellerica (Gaertn.) Roxb. (*T. bellerica*) is a plant in the family Combretaceae. The common names are Sa-Maw-Phi-Phek (Thailand), Belleric Myrobalan, and Belleric (India) (MOPH, 2000; Smitinand, 2001).

2.1 Botanical description

T. bellerica is a plant indigenous to Thailand and Southeast Asia. It is a deciduous tree with up to 40 – 50 m high. Its glabrous leaves are broadly elliptic to obovate with 10-24 cm x 5-8 cm and are clustered at the ends of the branchlets. Its flowers are small, yellowish-green and occur in axillary spikes. As shown in figure 2.2, it has a drupe, obscurely 5-angled fruit covered in soft hairs with a size of 2.5 x 2.0 cm, (MOPH, 2000; Smitinand, 2001).

2.2 Phytochemical studies

T. bellerica contains tannins such as chebulagic acid, ellagic acid, gallic acid, ethyl gallate (MOPH, 2000; Jadon, Bhadauria, & Shukla, 2007; Row et al., 1970; Smitinand, 2001). In addition, it consists of triterpeniods such as arjungenin, arjunglucoside, belleric acid, bellericaside A, bellericaside B, bellericoside (Nancy, Chakraborty, & Podder, 1997). It also composes β-sitosterol, mannitol, glucose, galactose, fructose, and rhamnose (Jadon, Bhadauria, & Shukla, 2007; Row et al., 1970). Its seeds have a new cardenolide, cannogenol 3-*O*-β-D-galactopyraosyl-(1→4)-*O*-α-L-rhamno-pyrano-side (Yadava & Rathore, 2001).

Figure 2.2

Terminalia bellerica (Gaertn.) Roxb.

2.3 Pharmacological studies

2.3.1 Antibacterial activity

An 80% ethanol extract from the fruits rind of *T. bellerica* is included in our antimicrobial screening program of Indian medicinal plants and is found to be active when screened against *E. coli*, *P. aeruginosa, S. aureus* and *B. subtilis* (Elizabeth, 2005). Moreover, alcohol extract from fruits exhibited antibacterial activity against *B. subtilis*, *P. vulgaris*, *S. typhimurium*, *E. coli*, *P. aeruginosa*, and *S. aureus* by using agar well diffusion method (Ahmad, Mehmood, & Mohammad, 1998).

2.3.2 Antifungal activity

An 80% ethanol extract of the fruit rind of *T. bellerica* and the isolation of termilignan, thannilignan, 7-hydroxy-3',4'-(methylenedioxy)flavan, and anolignan B exhibited antifungal activity against a number of pathogenic yeasts

Candida albicans and *Aspergillus niger* (Valsaraj et al., 1997).

2.3.3 Antiviral activity

The both methanol and water extracts from the dried fruit of *T. bellerica* displayed anti-HIV-1 reverse transcriptase (el-Mekkawy et al., 1995). A bioactivity-guided fractionation of an extract of *T. bellerica* fruit rind led to the isolation of two new lignans named termilignan and thannilignan, together with 7-hydroxy-3′,4′-(methylenedioxy)flavan and anolignan B. All four compounds possessed demonstrable anti-HIV-1 activity *in vitro* (Valsaraj et al., 1997).

2.3.4 Antimalaria activity

An 80% ethanol extract of the fruit rind of *T. bellerica* and the isolation of termilignan and thannilignan, 7-hydroxy-3',4'-(methylenedioxy)flavan, and anolignan B exhibited antimalarial activity against *Plasmodium falciparum* (Valsaraj et al., 1997).

2.3.5 Antioxidant activity

The 75% methanol extract from the fruit of *T. bellerica* inhibited lipid peroxide with Fe^{2+}/ascorbate and scavenged hydroxyl and superoxide radicals *in vitro*. The IC_{50} values of lipid peroxidation, hydroxyl radical scavenging and superoxide scavenging were 27, 71, and 40.5 μg/ml, respectively (Sabu & Kuttan, 2002). Furthermore, the extract from fruit showed high total phenolic contents (gallic acid and ellagic acid) and high antioxidant activity (Bajpai, Pande, Tewari, & Prakash, 2005).

2.3.6 Antidiabetic activity

An oral administration of 75% methanol extract from the fruit of *T. bellerica* (100 mg/kg body weight) significantly reduced the blood sugar level in normal and in alloxan (120 mg/kg) diabetic rats within 4 hours (Sabu & Kuttan, 2002).

2.3.7 Antimutagenic activity

An acetone extract from the fruit of *T. bellerica* displayed its effect against indirect acting mutagen, 2-aminofluorene, in both TA98 and TA100 tester stains of *S. typhimurium* rather than against the direct acting mutagens (Arora, Kaur, & Kaur, 2003).

2.4 Toxicological studies

2.4.1 Cytotoxicity

T. bellerica and *Phyllanthus emblica* extracts demonstrated growth inhibitory activity, with a certain degree of selectivity against the two cancer cell lines (human lung carcinoma; A549 and hepatocellular carcinoma; HepG2) tested. The growth inhibitory activity of doxorubicin or cisplatin, as a single agent, may be modified by combinations of *Phyllanthus emblica* and *T. bellerica* extracts and be synergistically enhanced in some cases. Depending on the combination ratio, the doses for each drug for a given degree of effect in the combination may be reduced. The mechanisms involved in this interaction between chemotherapeutic drugs and plant extracts remain unclear and should be further evaluated (Pinmai, Chunlaratthanabhorn, Ngamkitidechakul, Soonthornchareon, & Hahnvajanawong, 2008).

2.4.2 Renal and liver protective activities

The ethanol extract from the fruit of *T. bellerica* (doses 200, 400 and 800 mg/kg) and gallic acid (doses 50, 100 and 200 mg/kg) showed a dose dependent depletion in aspartate aminotransferase, serum alanine aminotransferase, serum alkaline phosphatase, and lipid peroxidation but a recovery of the glutathione level. These effects were more pronounced with gallic acid and treatment with gallic acid at the dose of 200 mg/kg was the most effective against carbon tetrachloride induced liver and kidney damage (Jadon, Bhadauria, & Shukla, 2007).

2.4.3 Acute toxicity

For *T. bellerica* in the acute toxicity test, an oral administration of the 50% alcoholic extract from its fruits demonstrated no toxic effects (convulsion, respiratory and cardiovascular pattern, morbidity, and mortality) at the dose of 10 g/kg body weight, but subcutaneous administration of this extract showed LD_{50} 6.15 g/kg body weight to mice. The amounts of *T. bellerica* recommended for the traditional medicine were 0.18 and 0.33 g/kg (Mokkhasmit et al, 1971). In addition, the oral administration of the 95% ethanol extract from its fruits in both female and male rats at the dose of 5 g/kg body weight showed no toxic effects on signs (convulsion, respiratory, motor activities, reflex, and gastrointestinal signs) and behavior, body weight, organ weight, and histopathology (Thanaporn, Jaijoy, Thamaree, Ingkaninan, & Panthong, 2006).

2.4.4 Subacute toxicity

The oral administration of the 95% ethanol extract from its fruits in rats at the dose of 1 g/kg body weight in subacute toxicity test showed no toxic effects on body weight, blood biochemistry (glucose, blood urea nitrogen (BUN), creatinine, albumin, total protein, total bilirubin, direct bilirubin, aspartate transaminase (AST), alanine transminase (ALT) and alkaline phosphatase (ALP), hematology (white blood cell (WBC), red blood cell (RBC), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), differential white blood cell count; lymphocytes, monocytes, neutrophil, eosinophil, and basophil), and relative organ weight, including histopathology of lung, heart, liver, spleen, adrenal gland, kidney, and sex organ (Thanaporn et al., 2006).

3. Toxicity study design

3.1 Acute toxicity

3.1.1 Definition (The Organization of Economic Co-operation and Development [OECD], 1981a; 2001)

Acute oral toxicity is the adverse effects occurring within a short time of oral administration of a single dose of a substance or multiple doses given within 24 hours.

Dose is the amount of test substance administered. Dose is expressed as weight (g, mg) or as weight of test substance per unit weight of test animal (mg/kg).

LD_{50} (median lethal dose), oral, is a statistically derived single dose of a substance that can be expected to cause death in 50 percent of animals when administered by the oral route. The LD_{50} value is expressed in terms of weight of test substance per unit weight of test animal (mg/kg).

Dosage is a general term comprising the dose, the frequency and the duration of dosing.

Dose-response is the relationship between the dose and the proportion of a population sample showing a defined effect.

Dose-effect is the relationship between the dose and the magnitude of a defined biological effect either in an individual or in a population sample.

3.1.2 Experimental animals (OECD, 1981a; OECD, 2001; Word Heath Organization [WHO], 2000)

3.1.2.1 Selection of species

Although several mammalian test species may be used, the rat is the preferred among rodent species. Commonly used laboratory strains should be employed. The weight variation in animals used in a test should not exceed ± 20 percent of the mean weight.

3.1.2.2 Number and sex

At least 10 rodents (5 female and 5 male) should be used for each dose level. The females should be nulliparous and non-pregnant.

3.1.2.3 Housing and feeding conditions

The temperature of the experimental animal room should be 22°C (± 3°C) and the relative humidity 30-70 percent. Animals may be group caged by sex, but the number of animals per cage must not interfere with clear observation of each animal. The biological properties of the test substance or toxic effects (e.g. morbidity,

excitability) may indicate a need for individual caging. The lighting is artificial and the sequence should be 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

3.1.3 **Test conditions** (OECD, 1981a; OECD, 2001)

3.1.3.1 Dose levels

These should be sufficient in number, at least three, and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose response curve and an acceptable determination of the LD_{50}.

3.1.3.2 Limit test

If a treatment of one dose level of at least 5,000 mg/kg body weight, using the procedures described for the study, produces no compound related mortality, then a full study using three dose levels may not be necessary.

3.1.3.3 Observation period

The observation period should be at least 14 days. However, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset and length of recovery period, and may be extended when it is considered necessary. The time at which signs of toxicity appear and disappear and the time of death are important, especially if there is a tendency to death to be delayed.

3.1.4 **Procedure** (OECD, 1981a; 2001)

Animals should be fasted prior to substance administration. For the rat, food should be withheld over-night. In the other rodents with higher metabolic rates, a shorter period of fasting is appropriate. Following the period of fasting, the animals should be weighed and then the test substance is administered in a single dose to animals by group by gavages using a stomach tube or a suitable intubation cannula. If a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours. After the substance has been administered, food may be withheld for a further 3-4 hours. Whereas a dose is administered in fractions over a period, it may be necessary to provide the animals with food and water depending on

the length of the period. Following administration, observations are made and recorded systematically with individual records being maintained for each animal.

3.1.5 **Clinical examinations** (OECD, 1981a; OECD, 2001)

A careful clinical examination should be made at least once each day. Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study, e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals. Cage side observations should be performed including changes in the skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, together with somatomotor activity and behavior pattern. Particular attention should be directed to observation of tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma. The time of death should be recorded as precisely as possible. Individual weights of animals should be determined shortly before the test substance is administered, weekly after the test and at death; changes in weight should be calculated and recorded when survival exceeds one day. At the end of the test surviving animals are weighed and then sacrificed.

3.1.6 **Pathology** (OECD, 1981a; 2001)

Necropsy of all animals should be carried out, and all gross pathological changes should be recorded. Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 or more hours should also be considered because it may yield useful information.

3.2 Chronic toxicity

3.2.1 **Definition** (Environmental protection agency [EPA], 1998)

Chronic toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by the oral, dermal, or inhalation routes of exposure.

Dose in a chronic toxicity study is the amount of test substance administered daily via the oral routes, dermal or inhalation routes for a period of at

least 12 months. Dose is expressed as weight of the test substance (g, mg) per unit body weight of test animal (mg/kg), or as weight of the test substance in parts per million (ppm) in food or drinking water per day. For inhalation exposure, dose is expressed as weight of the test substance per unit volume of air (mg/L) or as parts per million per day. For dermal exposure, dose is expressed as weight of the test substance (g, mg) per unit body weight of the test animal (mg/kg) or as weight of the substance per unit of surface area (mg/cm^2) per day.

3.2.2 Experimental animals (OECD,1981b; WHO, 2000)

3.2.2.1 Animal species

Testing should be performed with two mammalian species, one a rodent and the other a non-rodent. The rat is the preferred rodent species and the dog is the preferred non-rodent species. Commonly used laboratory strains should be employed. If other mammalian species are used, the tester should provide justification and reasoning for their selection.

3.2.2.2 Number and sex

Normally, the same number of male and female animals should be used. In cases of rodents, each group should consist of at least ten males and ten females. In the case of non-rodents, each group should consist of at least three males and three females. When interim examinations are scheduled, the number of animals should be increased accordingly.

3.2.2.3 Route of administration

The three main routes of administration are oral, dermal, and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in human.

3.2.2.4 Administration period

The period of administration of the test substance to animals will depend on the expected period of clinical use. The period of administration of the toxicity study may vary from country to country, according to its individual regulations. The following table reflects commonly used ranges of administration periods. As a rule, the test substance should be administered seven days a week.

Administration periods for the toxicity study must be recorded in each result (table 2.1).

Table 2.1

Commonly used ranges of administration periods

Expected period of clinical use	Administration periods for the toxicity study
Single administration or repeated administration for less than 1 week	2 weeks to 1 month
Repeated administration, between 1 week to 4 weeks	4 weeks to 3 months
Repeated administration, between 1 to 6 months	3 to 6 months
Repeated administration for more than 6 months	9 to 12 months

(WHO, 2000)

3.2.3 **Test condition** (OECD,1981b)

3.2.3.1 **Dose levels**

Groups receiving at least three different dose levels should be used. One dose level should not cause toxic changes (no-effect dose) and one dose level that produces overt toxic effects should be included. Within this range the addition of at least one more dose may enhance the possibility of observing a dose response relationship for toxic manifestations. All studies should include a vehicle control group of test animals.

3.2.3.2 **Duration of study**

The duration of the exposure period should be at least 12 months.

3.2.4 **Observations and examinations** (OECD,1981b; WHO, 2000)

3.2.4.1 **General signs, body weight, food and water intake**

For all experimental animals, the general signs should be observed daily whereas the body weight and food intake should be measured periodically. If useful, water intake should also be determined. Measurements of body weight and food intake should be perform as follows: before the start of drug administration, at

least once a week for the first three months of administration and at least once every four weeks thereafter.

3.2.4.2 Hematological examination

Hematological examination (e.g. hemoglobin content, packed cell volume, total red blood cells, total white blood cells, platelets, or other measures of clotting potential) should be performed at 3 months, 6 months, and at approximately 6 months intervals thereafter and at termination on blood samples collected from all non-rodents and from 10 rats per sex of all groups. If possible, these collections should be achieved from the same rats at each interval. In addition, a pre-test sample should be collected from non-rodents as well.

If clinical observations suggest deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed.

A differential blood count is performed on samples from those animals in the highest dosage group and the control group. Differential blood counts are performed for the next lower group(s) only if there is a major discrepancy between the highest dosage and the control, or if there is an indication from the pathological examination.

3.2.4.3 Biochemical examination

At approximately 6 month intervals, and at termination, blood samples are drawn for biochemical measurements from all non-rodents and 10 rats per sex of all groups, if possible, from the same rats at each interval. In addition, a pre-test sample should be collected from non-rodents. Plasma is prepared from these samples and the following determinations are made in order to determine total protein concentration, albumin concentration, liver function test, carbohydrate metabolism, and kidney function test.

3.2.4.4 Gross necropsy

All animals in the study should normally be subjected to a full, detailed gross necropsy, which includes careful examination of the external surface of the body, all orifices, together with the cranial, thoracic and abdominal cavities and their contents.

Organ weights should be collected from all animals. The adrenals, brain, epididymis, heart, kidneys, liver, ovaries, spleen, testes, thyroid, and uterus of all animals (apart from those found moribund and/or intercurrently killed) should be trimmed of any adherent tissue, as appropriate, and their wet weight should be taken as soon as possible after dissection to prevent drying. In the case of paired organs, e.g., kidney, adrenal, both organs should be weighed separately.

The following tissues should be preserved in the most appropriate fixation medium and the intended subsequent histopathological examination: all gross lesions, adrenal gland, aorta, brain, caecum, cervix, colon, duodenum, epididymis, eye, heart, ileum, jejunum, kidney, lacrimal gland, liver, lung, lymph nodes, female mammary gland, esophagus, ovary, pancreas, parathyroid gland, peripheral nerve, pituitary, prostate, salivary gland, seminal vesicle, skeletal muscle, skin, spinal cord, spleen, stomach, testis, thymus, thyroid gland, trachea, urinary bladder, uterus, vagina, and a section of bone marrow.

3.2.4.5 Histopathological examinations

The minimum histopathological examination should be evaluated in all tissues from the high dose and control groups. However, examination is also determined in following tissues composed of all tissues from animals dying or killed during the study, all tissues showing macroscopic abnormalities, target tissues, or tissues which showed treatment-related changes in the high dose treatment group, together with tissues from all animals in all other treatment groups. Paired organs such as kidney, adrenal, both organs should be examined.

3.2.4.6 Recovery from toxicity

In order to investigate the recovery from toxic changes, animals which are allowed to live for varying lengths of time after cessation of the period of administration of the test substance, should be examined.