CHAPTER II

REVIEW OF RELATED LITERATURES

2.1 *Phyllanthus amarus* Schum. et. Thonn.

2.1.1 General description

Phyllanthus amarus Schum. et. Thonn. is a plant in the family Euphorbiaceae (previously known as *Phyllanthus niruri* Linn.) (Khatoon *et al.*, 2006; Rajeshkumar and Kuttan, 2000). It is mostly grown as weed in open ground, agricultural and waste lands and is widespread during the rainy season. (Rai *et al.*, 2005). It is distributed in tropical and subtropical countries such as India, China, Pakistan, Cuba, Indonesia, Brazil, Nigeria, East and West Africa, the Philippines, Tanzania and Thailand. This plant has unique character as fruits underneath its leaf similar to other plants in the genus *Phyllanthus* which found in Thailand such as *Phyllanthus debilis* Klein & Willd, *Phyllanthus urinaria* Linn. and *Phyllanthus virgatus* Forst.f. However, the differences can be identified between species using characteristic of stem, branch and fruit, color of leaf and young leaf and the number of petal and sepal. (จารีย์ บัณสิทธิ์, 2534; พิศมัย เหล่าภัทรเกษม และคณะ, 2544).

P. amarus was found in all regions of Thailand with different local names such as Luk-tai-bai (ลูกใต้ใบ), Ma-Kham-Pom-Din (มะขามป้อมดิน) and Yah-tai-bai-Khaow (หญ้าใต้ใบขาว) (ป่าไม้, กรม, 2544).

2.1.2 Botanical description

Phyllanthus amarus is an annual, erect, slender, branching, smooth herb that grows up to 10 to 60 cm tall. The stem and branches are rather round, smooth and light green in color. Its leaves are simple, elliptic belong to obovate, 2 to 6 mm wide and 4-11 mm long, round or obtuse at the apex, and obtuse at the base. The leaf margin is smooth, no ciliate, rather pale underneath, and with a short petiole, 0.2 to 0.5 mm long. Male and female flowers are separate. Male flowers are very minute. It has 2 to 3 flower and 1 to 4 lowest axils, with short peduncles about 1 mm (they have 5 oval-shaped petal) about 0.2 to 0.5 mm wide and 0.3 to 0.6 mm long (with 3 connate stamens). Female flowers are twice as large, solitary in higher axil,

accompanied by a few male ones, (with 5 oval shaped petal) about 0.4 to 0.6 mm wide and 0.5 to 1 mm long. The disk is saucer-shaped with 5 toothed smooth capsules, 3-celled, two smooth seeds in each cell, 3-short styles and free bifid. The fruit is small, flatted round, with a diameter of about 1 to 1.5 mm, light green, smooth, with 6 minute seeds of triangle shape and 5 to 6 longitudinal ribs (จารีย์ บัณสิทธิ์, 2534; Kongstan, 2000).

2.1.3 Chemical constituents

Phyllanthus amarus has several chemical constituents as follows:

1.) Lignans such as phyllanthin, hypophyllanthin, lintetralin, isolintetralin, hydroxyniranthin, nirtetralin, demethylenedioxy niranthin, 2,3-desmetoxy seco-isolintetralin, seco-4-hydroxy lintetralin, linnanthin, seco-isolariciresinol, nirphyllin trimethylether and phyllnirurin.

2.) Flavonoids such as nirurin, nirurinetin, kaemferol-4-rhamnopyranoside, eriodictyol-7-rhamnopyranoside, quercetin, isoquercitin, astragalin, rutin, catechin and epigallocatechin.

3.) Steroids and Triterpenoid such as phyllanthenol, phyllanthenone, lupeol, phyllantheol, β-sitosterol and 24-isopropyl-cholesterol.

4.) Alkaloids such as nirurine, securinine, norsecurinine, ent-norsecurinine, isobubbialine, phyllanthine (4-methoxy-securinine), 4-methoxy-nor-securinine and epibubbialine.

5.) Tannins such as repandusinic acid A, amariinnic acid, amariin, geraniin, amarulone, geraniinic acid B, elaeocarpusin, phyllanthusiin D, corilagin, ellagic acid and gallic acid.

6.) Other constituents such as niruriside, triacontanal, triacontanol, ricinoleic acid, phyllester and dotriacontanoic acid.

Moreover, it also has the high content of potassium in this herb.

(นิจศิริ เรืองรังษี และพยอม ตันติวัฒน์, 2532; Kumar and Kuttan, 2004; Teerasukaporn, 1998).

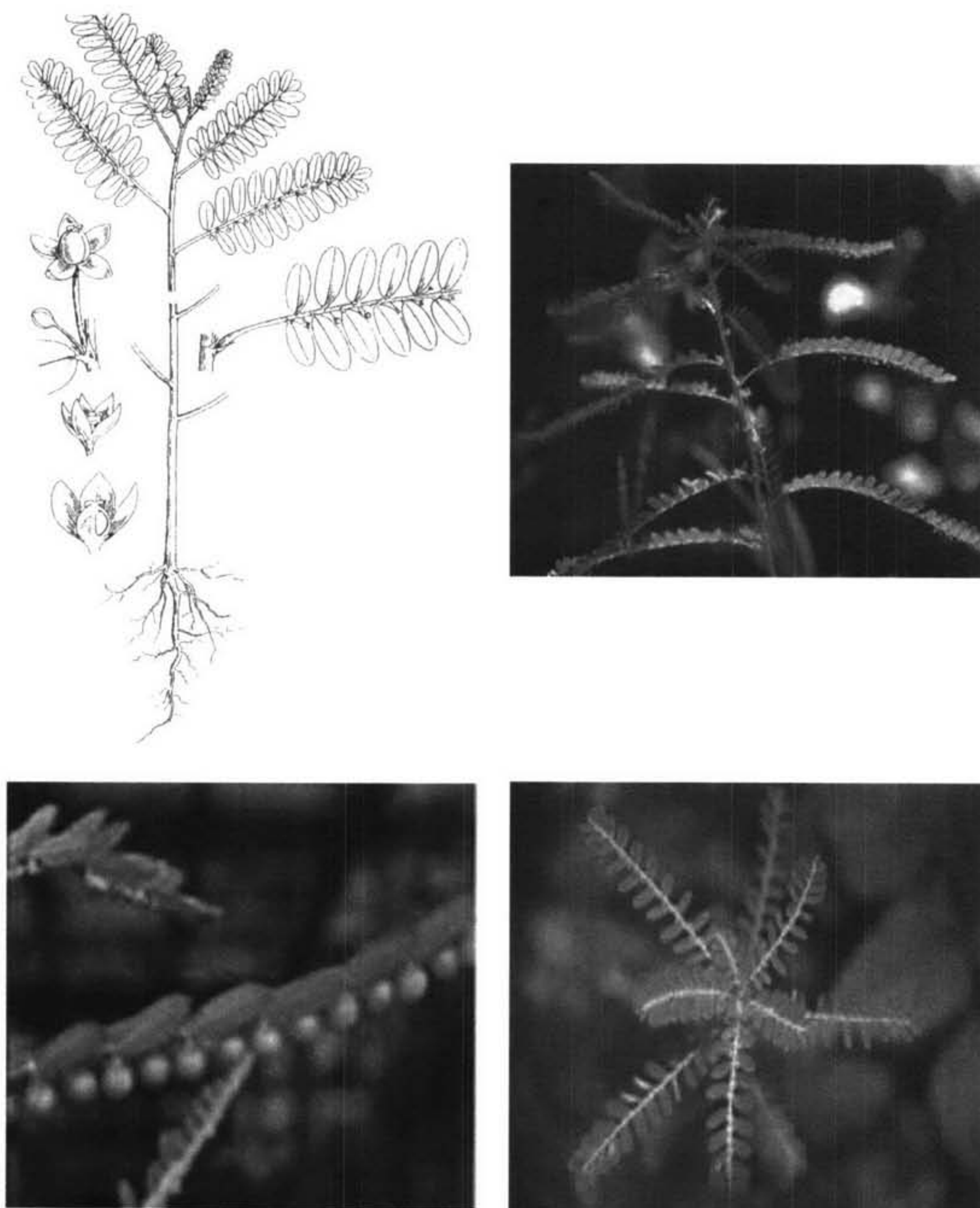


Figure 1. *Phyllanthus amarus* Schum. et. Thonn. (Thai as "Luk tai bai")
(Khare, 2004)

2.1.4 Traditional medicinal uses

Thailand: whole plant is used for antipyretic, treatment of stomach pain, venereal diseases, jaundice, hemorrhoid and antidysenteric, young leaf is used for cough remedy for children (มหาวิทยาลัยมหิดล, คณะเภสัชศาสตร์, 2535). The plant decoction is used for emmenagogue, diuretic and bitter tonic (นิจศิริ เรืองรังษี และพยอม ตันติวัฒน์, 2532).

Other countries:

- Tanzania: aqueous extract of the aerial part is used for treatment of diabetes mellitus, the leaves are chewed against persistent coughs and stomachaches.

- India: whole plant is used for treatment of hepatitis, dysentery, irritating sores, jaundice and diuretic.

- The West Indies: whole plant is used for prevention of intestinal worms in children.

- Rarotonga (Cook Islands): the entire plant is used for treatment of earache.

- Papua New Guinea: the fresh whole plant decoction is drunk to treat migraine.

- Nigeria: water extract of the dried whole plant is used for treatment of diarrhea.

- China: the plant decoction is used to promote urination and to dissolve bladder stones.

- Malaysia and Indonesia: the plant is used to promote expectoration, reduce fever, to promote menstruation, stop diarrhea and to reduce inflammation and to stimulate children appetite (Moshi *et al.*, 2001; Wong, 2002).

2.1.5 Pharmacological and toxicological effects

1.) Antihepatotoxic effect

1.1) Antihepatotoxic effect against paracetamol induced liver injury

Aqueous extract at doses of 3, 30 and 300 μg/ml reduced the percent leakage of Lactate dehydrogenase (LDH) in 5 mM paracetamol treated primary cultured rat hepatocytes (พิศมัย เหล่าภัทรเกษม และคณะ, 2544). An oral single dose of hot water extract of whole plant (0.8-1.5 g/kg) given 4 hours before single dose of

paracetamol (3 g/kg) in rats showed antihepatotoxic effect, but administration of this extract after paracetamol 4 hours or administration with dried powder suspension had no effect in reduction of hepatotoxicity from paracetamol (วันดี อุดมอักษร, 2543). In addition, administration of hot water extract at doses of 1.6 and 3.2 g/kg orally for 7 days before single oral dose of paracetamol (3 g/kg) were actively hepatoprotective by reduction of serum glutamic oxaloacetic transminase (SGOT) or aspartate aminotransferase (AST), serum glutamic pyruvic transminase (SGPT) or alanine aminotransferase (ALT), serum bilirubin and histopathological changes. The hepatoprotective effect of this extract was partly due to the antioxidant activity and the protective effect on the decrease in hepatic glutathione (Wongnava *et al.*, 2000).

1.2) Antihepatotoxic effect against carbon tetrachloride (CCl_4) induced liver injury

Aqueous extract of *P. amarus* (90 mg/kg), Phyllanthin (a bioactive lignan from *P. amarus*) and Narocil (a tablet of aqueous extract of *P. amarus*) showed hepatoprotective effect against CCl_4 induced hepatotoxic rats (Venkatesan *et al.*, 2003). Aqueous extract at a dose of 0.1 g/kg given by intraperitoneal injection (i.p.) 30 minutes before CCl_4 in mice showed the reduction of ALT level (พิศมัย เหล่าภัทรเกษม และคณะ, 2544).

Ethanolic extract at doses of 40 and 400 mg/kg for 7 days and of 5 g/kg for 28 days given orally before CCl_4 in rats showed hepatoprotective effect by reduction of SGOT, SGPT and damage of hepatocytes (Prakash *et al.*, 1995; Walaiphachara, 1994). Methanolic extract and aqueous extract of whole plant at a dose of 100 mg/kg given orally before CCl_4 in rats showed reduction of SGOT, SGPT and lipid peroxides levels (Harish and Shivanandappa, 2005). Furthermore, chemical constituents are phyllanthin and hypophyllanthin which isolated from hexane extract were active against CCl_4 induced cytotoxicity in primary cultured rat hepatocytes (Syamasundar *et al.*, 1985).

1.3) Antihepatotoxic effect against galactosamine (GalN) induced liver injury

Ethanolic extract at doses of 2.5, 5 and 10 g/kg given orally for 28 days before GalN in rats reduced level of SGOT, SGPT, the number of necrotic

hepatocytes and inflammatory cell infiltration. This extract caused no changes in aniline hydroxylase, UDP-glucuronyltransferase activity. It does not induce or inhibit cytochrome P450 2E1 activity and glucorunidation. (Kongstan, 2000). Furthermore, its chemical constituents, phyllanthin, hypophyllanthin and triacontanal which isolated from hexane extract were active against GalN induced cytotoxicity in primary cultured rat hepatocytes (Syamasundar *et al.*, 1985).

1.4) Antihepatotoxic effect against ethanol induced liver injury

Whole plant powder at a dose of 200 mg/kg given to rats for 45 days after ethanol administration for 30 days showed the decrease in deposition of hepatic triglyceride, cholesterol and phospholipids (Umarani *et al.*, 1985).

2.) Antiviral effect

2.1) antihepatitis B virus (Anti-HBV)

Aqueous extract at a single dose of 1 mg/ml showed inhibition of hepatitis B surface antigen (HBs Ag) secretion from Alexander cell line (Jayaram and Thyagarajan, 1996).

Aqueous extract inhibited cellular proliferation and suppressed HBsAg production in HepA2 cells. This study suggested that the extract contains some active components which suppressed HBsAg gene expression at mRNA level (Yeh *et al.*, 1993).

Aqueous extract showed anti-HBV by inhibition of binding between HBsAg and anti-HBsAg with the 50% inhibitory concentrations (IC_{50}) of 1,682 ng/100 µl (บังอร ศรีพานิชกุลชัย และคณะ, 2544) or 959 ng/100 µl (พิศมัย เหล่าภัทรเกษม และคณะ, 2544).

Administration of whole plant powder in capsule at a dose of 200 mg, three times a day for 30 days on chronic carriers of HBV showed the 59% lost of HBsAg, when follow-up to 9 months there was no case of antigen reappearing and no toxic effect (Thygarajan *et al.*, 1988).

P. amarus compound treated on chronic viral hepatitis B patients for 3 months showed the total effective rate of 83.3% similar to interferon alpha- 1b. Both of them had effect on recovery of liver function and inhibition of the replication of HBV (Xin-Hua *et al.*, 2001).

The possible mechanism of anti-HBV of this herb may be due to several mechanisms as follows: (1) Inhibition of HBV mRNA transcription by interrupting

the interactions between HBV enhancer I and cellular transcription factors (Ott *et al.*, 1997). (2) Inhibition of HBsAg and anti-HBsAg binding (บังอร ศรีพานิชกุลชัย และคณะ, 2544; พิศมัย เหล่าภัทรเกษม และคณะ, 2544). (3) Inhibition of HBV polymerase (Lee *et al.*, 1996; Blumberg *et al.*, 1989).

On the other hand, there are many reports showed that this herb had a very minimal or no effect as an anti-HBV (Chiannuvati *et al.*, 1994; Doshi *et al.*, 1994; Narendranathan *et al.*, 1999; Thamlikitkul *et al.*, 1991).

Furthermore, aqueous extract showed an inhibition on woodchuck hepatitis viruses (Venkateswaran *et al.*, 1987), while butanolic and ethanolic extract at doses of 25, 50 and 200 g/kg had no anti-duck hepatitis B virus (Munshi *et al.*, 1993).

2.2) Anti-human immunodeficiency virus (Anti-HIV)

Repandusinic acid A was isolated from aqueous extract showing the inhibition on HIV type 1 reverse transcriptase (HIV-1-RT) and DNA polymerase alpha with IC_{50} of 0.05 and 0.6 mM, respectively (Ogata *et al.*, 1992).

Aqueous, aqueous/ethanolic and methanolic extract inhibited HIV-1 replication in HeLa $CD4^+$ cells with the effective concentration (EC_{50}) from 0.9-7.6 μg/ml. A gallotannin enriched fraction, geraniin and corilagin (the purified gallotannins) had EC_{50} of 0.4 and 0.24 μg/ml, respectively. Aqueous/ethanolic extract and geraniin showed 70-75% of virus uptake at concentration of 2.5 μg/ml. In addition, all of the above extracts showed inhibition of HIV-1-RT with IC_{50} from 1.8 to 14.6 μg/ml (Notta *et al.*, 2003).

Furthermore, aqueous/ethanolic extract, gallotannin enriched fraction, geraniin and corilagin blocked HIV-1 attachment by blockade the interaction of HIV-1 gp120 and CD4 recepter and inhibited the HIV-1 enzymes integrase, reverse trancriptase and protease. Administration of aqueous/ethanolic extract at a dose of 1200 mg orally to volunteers showed reduction of HIV-1 replication by more than 30% (Notta *et al.*, 2004).

Alkaloidal extract showed inhibition of HIV-1 replication on human MT-4 cells (Naik and Juvekar, 2003)

Niruriside, isolated from methanolic extract, showed anti-HIV by inhibition against the binding of HIV-regulation of virion expression protein (HIV-REV) to REV-responsive element (RRE) (Cutrone *et al.*, 1996).

2.3) Anti-herpes simplex virus

Aqueous/ethanolic fraction, ethyl acetate fraction and aqueous/ethyl acetate fraction were active against herpes simplex virus type 2 (HSV-2) strain Baylor 186 on Vero cell lines (Wirotsangthong *et al.*, 2002).

3.) Antibacterial and antifungal effects

Aqueous extract had antibacterial effect with minimum bactericidal concentration (MBC) at 3.91 mg/ml for *Staphylococcus aureus,* at 31.25 mg/ml for *Pseudomonas aeruginosa and Shigella species,* and at 62.5 mg/ml for *Escherichia coli* (บังอร ศรีพานิชกุลชัย และคณะ, 2544).

Ethanolic extract of aerial part showed antibacterial effect against *Bacillus cereus, Bacillus subtilis, Bacteroides fragilis, Enterococcus faecalis, Pseudomonas aeruginosa*, *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli*, and *Streptococus pyogenes* with minimum inhibitory concentrations (MIC) ranging from 0.25 to 16 mg/ml (Kloucek *et al.*, 2005). Methanolic, ethanolic and acetone extracts inhibited *B. cereus* and *S. aureus* (Kernsakphai *et al.*, 1998).

Furthermore, methanolic extract of leaf inhibited the growth of *S. aureus, S. pyogenes, E. coli* and *Proteus spp.*; and displayed antifungal effect against *Candida albicans* (Onocha *et al.*, 2003).

4.) Antimutagenic, anticarcinogenic and antitumor effects

Methanolic extract at doses of 0.25-2 mg/plate showed inhibition of the activation and mutagenicity of 2-acetaminofluorene (2-AAF) and aflatoxin B, at doses of 1-0.25 mg./plate showed inhibition of mutagenicity induced by direct acting mutagens sodium azide (NaN_3), N-methyl-N-Nitro-N-nitrosoguanidine (MNNG) and 4-nitro-o-phenylenediamine (NPD) in Salmonella typhimurium stains TA 1535, TA 100 and TA 102. Oral administration of this extract to rats showed inhibition of urinary mutagenicity induced by benzopyrene (Raphael *et al.*, 2002).

Crude extract at doses of 0.25, 0.5, 0.75 and 1 % showed reduction in the frequency of chromosomal alterations in the tannery effluents induced genotoxicity which suggested that phyllanthin is antigenotoxicant (Gowrishanker and Vivekanandan, 1994).

Aqueous extract of whole plant showed antimutagenic effect against 2-aminofluorene, 2-aminoanthracene, 4-nitroquinoline-1-oxide, 2-nitrofluorene, N-ethyl-N-Nitro-nitrosoguanidine and sodium azide induced mutagenicity in

Salmonella typhimurium Strains TA 98 and TA 100 and *Escherichia coli* WP2 uvrA/PKM101. In addition, this extract at an oral dose of 2 g/kg to hamster showed anticarcinogenic effect against dimethylnitrosamine induced DNA single-strand breaks in hamster liver cells. Both effects may be due to gallotannins which are the major constituents in aqueous extract (Sripanidkulchai *et al.*, 2002).

Aqueous extract of aerial part at doses of 60, 300 and 1500 mg/kg inhibited the sarcoma development induced by 20-methylcolanthrene, increased the life span of sarcoma bearing mice and reduced the volume of transplanted solid tumors. Furthermore, this extract inhibited aniline hydroxylase, a P450-enzymes (IC_{50} of 540 μg/ml), inhibited DNA topoisomerase II of *Saccharomyces cerevisiae* mutant cell cultures (IC_{50} of 250 μg/ml) and inhibited cell cycle regulatory enzyme cdc25 tyrosine phosphatase (IC_{50} of 25 μg/ml). Antitumor and anticarcinogenic effect of this extract may be mediated by the inhibition of cell cycle regulation, topoisomerase II, P450-enzymes and oxygen radicals (Rajeshkumar *et al.*, 2002).

Aqueous extract at doses of 150 and 750 mg/kg showed inhibition of hepatocarcinogenesis induced by N-nitrosodiethylamine (NDEA) in rats. This extract inhibited tumor incidence and reduced levels of carcinogen metabolizing enzymes (glutathione S-transferase (GST) and aniline hydroxylase), levels of liver cancer markers (γ-glutamyl transpeptidase (GGT) and reduced glutathione (GSH)) and levels of liver injury markers (lipid peroxide (LPO), alkaline phosphatase (ALP), glutamate pyruvate transminase (GPT)) and total bilirubin. The above effects may be due to the action of polyphenols likes ellagic acid or flavonoids or lignans in extract. (Jeena *et al.*, 1999). In addition, the same preparation and doses increased the life span of rats with hepatocellular carcinoma induced by NDEA by 54.48 % (Rajeshkumar and Kuttan, 2000).

Ethanolic extract of whole plant showed inhibition of the proliferation of cancer cells such as Hep G2 (hepatic cancer), SKOV3 (ovarian cancer), SKBR3 (breast cancer) and BT549 (breast cancer) (Kosem *et al.*, 2000).

Methanolic extract of aerial parts at doses of 250 and 750 mg/kg administered to mice induced toxicity by cyclophosphsmide (CTX) showed the decrease in activity of phase I enzyme (aniline hydroxylase) and the increase in Phase II enzyme (GST) and GSH, thereby decreasing the effect of toxic metabolites of CTX. This extract also had the synergistic action with CTX in reducing the

tumor, and no interfering with antitumor efficiency of CTX (Kumar and Kuttan, 2005).

5.) Antioxidant effect

Methanolic extract showed inhibition of lipid peroxidation (IC_{50} of 104 μg/ml) and scavenging of hydroxyl and superoxide radicals (117 and 19 μg/ml, repectively) (Raphael, Suba and Kuttan, 2002).

Methanolic and aqueous extract of leaves and fruits showed inhibition of membrane lipid peroxidation (IC_{50} of 42.3-204 μg/ml), scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (IC_{50} of 9.1-32.6 μg/ml) and inhibition of superoxide production (IC_{50} of 0.1-2.81 μg/ml) (Harish and Shivanandappa, 2006).

Oral administration of methanolic extract of aerial parts at a dose of 750 mg/kg/day to mice for 5 days before the whole body radiation with 6 Gy of cobalt 60 and at a dose of 250 mg/kg/day orally for 1 month after radiation. Results showed the increase in the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione-s-transferase (GST), glutathione peroxidase (GPX) and glutathione reductase (GR) in blood and tissue. In addition, there were the increase in glutathaione (GSH) levels and the decrease in lipid peroxidation (Kumar and Kuttan, 2004).

Furthermore, this herb showed Nitric Oxide (NO) scavenging activity in sodium nitroprusside as NO donor in vitro (Jagatia and Baliga, 2004).

6.) Anti-nociceptive and anti-inflammatory effects

Hydroalcoholic extract of leaves, stem and roots at doses of 0.3-100 mg/kg intraperitoneally, 30 minutes and at doses of 10-400 mg/kg orally, 60 minutes before acetic acid showed inhibition of acetic acid induced abdominal constrictions, At doses of 0.3-300 mg/kg, intraperitoneally and doses of 100-800 mg/kg orally showed inhibition of formalin induced licking. At doses of 3-60 mg/kg intraperitoneally and doses of 200-900 mg/kg orally showed inhibition of capsaicin induced neurogenic pain (Santos *et al.*, 2000).

Hexane extract of aerial parts at a dose of 100 mg/kg orally showed inhibition of the allodynia and edema induced by the intraplantar injection of complete Freund's adjuvant (CFA) and inhibition of the mechanical allodynia caused by partial ligation of scialic nerve in mice (Kassuya *et al.*, 2003).

Methanolic and aqueous extract at doses of 19 and 700 mg/kg, respectively showed inhibition of abdominal constrictions induced by acetic acid (พิศมัย เหล่าภัทรเกษม และคณะ, 2534; Santos *et al.*, 1994).

Aqueous extract of whole plant at doses of 360 and 700 mg/kg intraperitoneally and of leaves and stems at doses of 100, 250 and 500 mg/kg orally, and methanolic extract of leaves and stems at doses of 100, 250 and 750 mg/kg orally showed anti-inflammatory effect in carrageenan induced mice paw edema. The mechanism of this effect may be due to the inhibition of macrophage migration, prostaglandin synthesis and production of oxygen radical (พิศมัย เหล่าภัทรเกษม และคณะ, 2534; Raphael and Kuttan, 2003).

Ethanol/aqueous and hexane extract showed inhibition of lipopolysaccharide (LPS) induced the production of nitric oxide and prostaglandin E_2 (PGE_2) in rat kupffer cells and RAW 264.7 macrophages, inhibition of the production of TNF-α in RAW 264.7 macrophages and human whole blood, inhibition of the production of IL-1β, IL-10 and interferon-γ (IFN-γ) in human whole blood and reduction of the production of TNF-α in galactosamine and LPS induced acute toxic hepatitis mice. Furthermore, both extracts reduced expression of nitric oxide synthase (iNOS), cyclooxygenase (COX-2) and inhibited activation of NF-kappa B (Kiemer *et al.*, 2003).

7.) Inhibition of gastric lesions

Methanolic extract of leaves and stems at doses of 50, 200 and 1000 mg/kg inhibited gastric lesions of rats treated with absolute ethanol (8 ml/kg) by reduction of mortality, increasing stomach weight, ulcer index and gastric mucosa GSH. The possible mechanism of this extract may be due to the formation of a protective layer by the polyphenolic compounds in the extract with stomach protein lining by hydrophobic interaction and the reduction of oxygen radical production by tannin in the extract (Raphael and Kuttan, 2003).

8.) Antidiarrheal effect

Aqueous extract of leaves at doses of 100-800 mg/kg orally showed a dose-related inhibition of gut meal travel distance in normal mice. This extract (400 mg/kg) delayed the onset of diarrhea, reduced frequency of defecation and gut meal

travel distance, resulting in intestinal transit inhibition of 79.94% (Odetola and Akojenu, 2000).

9.) Hypoglycemic effect

Methanolic extract at doses of 200 and 1000 mg/kg showed reduction in blood sugar by 6 and 18.7%, respectively after given for 4 hours in alloxan induced hyperglycemic rats. When given this extract at a dose of 1000 mg/kg for 18 consecutive days, the reduction in blood sugar was similar to normal rats (Raphael, Suba and Kuttan, 2002).

Aqueous extract at doses of 0.1 and 1 g/kg enhanced the clearance of an oral glucose load of normal rabbits but did not lower fasting blood glucose (FBG) (Moshi *et al.*, 1997).

Nine human subjects were treated with a whole plant preparation for 10 days showed the reduction of blood glucose and revealed no harmful side effects (Srividya and Periwal, 1995).

However, administration of aqueous extract of aerial parts at a dose of 12.5 g twice daily for 7 days in non-insulin dependent diabetic (NIDDM) patients showed incapable of lowering either FBG or postprandial bloo glucose (Moshi *et al.*, 2001).

10.) Hypotensive effect

Nine human subjects were treated with a preparation of whole plant for 10 days showed hypotensive effect by reduction in systolic blood pressure and increasing in urine volume (Srividya and Periwal, 1995).

11.) Contraceptive effect

Ethanolic extract of whole plant at a dose of 500 mg/kg orally to male mice for 45 days showed inhibition of fertility rats by 72% and reduction of sperm motility and viability. This results were gradually reversible after the withdrawal of plant feeding (Rao, Shah, and Rajani, 1997).

Aqueous extract of leaves at a dose of 400 mg/kg orally to rats for 14 days caused testicular degeneration and reduction in the mean seminiferous tubular diameter (Adedapo *et al.*, 2003).

Ethanolic extract of whole plant at a dose of 100 mg/kg orally to female mice for 30 days showed reduction of 3β and 17β hydroxy steroid dehydrogenase

(HSDs) levels. After the withdrawal of plant feeding for 45 days, these effects were reversible (Rao and Alice, 2001).

Furthermore, in the study of sex hormones found that whole plant had high content of estradiol (Mannan and Ahmad, 1978).

12.) Inhibition of renal stone formation and Diuretic effects

Aqueous extract showed inhibitory effect on the Calcium Oxalate (CaOx) crystal endocytosis by renal tubular cells (Campos and Schor, 1999) and inhibition of CaOx crystals growth and aggregation in human urine (Barros, Schor, and Boim, 2003).

Rats were induced renal stone formation by the introduction of a CaOx seed into the bladder, the extract at a dose of 1.25 mg/ml/day orally for 45 days showed inhibitory effect on crytal growth (Freitas, Schor and Boim, 2002).

Administration of extract at a dose of 400 mg/day for 3 month in calcium stone forming (CSF) patients showed reduction of urinary calcium (Nishiura *et al.*, 2004).

For the diuretic effect, aqueous extract at an oral dose of 700 mg/kg, 30 minutes before bicarbonate saline induced diuretic rats showed no effect on urine volume while intraperitoneal administration showed the decrease in urine volume (พิศมัย เหล่าภัทรเกษม และคณะ, 2534).

13.) Antipyretic effect

Aqueous extract of whole plant at a dose of 700 mg/kg intraperitoneally showed antipyretic effect in typhoid vaccine induced pyretic rats (พิศมัย เหล่าภัทรเกษม และคณะ, 2534).

14.) Lipid lowering effect

Crude extract at doses of 100 and 250 mg/kg showed reduction of lipid and apoprotein of VLDL and LDL levels in triton and cholesterol induced hyperlipemic rats. This effect was mediated through inhibition of hepatic cholesterol synthesis, increased faecal bile acids excretion, enhaned plasma lecithin: cholesterol acyltransferase (LCAT) activity and tissue lipases (Khanna, Rizvi and Chandar, 2002).

15.) Antimalarial effect

Ethanolic extract, dichloromethane extract, aqueous extract, petroleum ether soluble fraction and isoamyl alcohol fraction of whole plant showed inhibition of *Plasmodium falciparum* growth in vitro (Subeki *et al*., 2005; Tona *et al*., 1999; Tona *et al*., 2004).

Ethanolic, dichloromethane and aqueous extract of whole plant at a dose of 200 mg/kg showed reduction of parasitaemia against *Plasmodium berghei ANKA* in mice (Tona *et al*., 2001).

Moreover, Ethanolic extract from fresh apical stem exhibited lower inhibition of *Plasmodium falciparum* infection than ethanolic extract of whole plant (Cimanga *et al*., 2004).

16.) Angiotensin-converting enzyme inhibition

Geraniin isolated from whole plant at a dose of 100 mg/ml showed inhibition of angiotensin-converting enzyme (Ueno *et al*., 1988).

17.) Aldose reductase inhibition

Ellagic acid, brevifolin carboxylic acid and ethyl brevifolin carboxylate isolated from the 70% ethanolic extract showed inhibition of aldose reductase enzyme with ellagic acid had the highest inhibitory effect (Shimizu *et al*., 1989).

18.) Toxicological effect

Ethanolic extract at doses of 2.5, 5 and 10 g/kg orally for 28 days showed no effect on growth rates, food consumption and toxic injury in rats (Kongstan, 2000) However, at a dose of 25 g/kg had a decrease in growth rates and food consumption, the level of blood urea nitrogen (BUN), total protein and plasma glucose were higher than control rats (Walaiphachara, 1994).

Aqueous extract of the leaves at doses of 400, 800 and 1000 mg/kg orally for 30 days showed the decrease in red blood cell (RBC) count, packed cell volume (PCV), haemoglobin (Hb) concentration and the increase in white blood cell (WBC) count and the level of AST, total and conjugated bilirubin, total protein and albumin showing toxic effect on liver, kidney and testes (Adedapo, Adegbayibi and Emikpe, 2005).

2.2 Liver

Liver is the largest organ in mammalian body, about 3.5% body weight of an adult rat or 2% body weight of an adult human. It locates at the top of the abdomen, on the right side of the body just below the diaphragm. In rats and mice, liver is divided into several distinct lobes (Ballantyne, Marrs, and Syversen, 2000). However, in human, it is divided into two main lobes (Sherlock and Dooley, 2002). Liver has many functions that important to body as follows:

1. Production and secretion of bile into the intestine for facilitating emulsification and absorption of dietary fat.

2. Regulation of carbohydrate metabolism by removing glucose from blood when blood glucose levels are high (glucose to glycogen by glycogenesis) and when blood glucose levels are low (glycogen to glucose by glycogenolysis). Furthermore, it is also responsible for converting lipids, amino acids and lactates into glucose by gluconeogenesis.

3. Regulation of protein metabolism by synthesis and secretion of protein such as albumin, clotting factor, globulin. Moreover, it is the site of amino acid deamination and removal of ammonia by synthesis of urea which excreted by kidney.

4. Regulation of lipid metabolism by synthesis of lipoproteins, transport lipid into plasma, storage and secretion of triglyceride, and regulating uptake and excretion of cholesterol.

5. Storage of vitamins such as vitamin A, D, E, K and B_{12}.

6. Metabolism, detoxification and inactivation of both exogenous compounds, such as drugs and toxic substances, and endogenous compounds, such as steroids and other hormones. (ชัยวัฒน์ ต่อสกุลแก้ว, 2541; Gitnick, Labrecque and Moody, 1991; Junqueira and Carneiro, 1983).

Liver contains many types of cells. The main structural component is the parenchymal cell or liver cell or hepatocyte which make up about 60% of the liver cell population with a diameter of approximately 20-30 μm (Junqueira and Carneiro, 1983), In the hepatocytes (Figure 2), the nucleus is normally placed center and the cytoplasm consists of mitochondria (20%), peroxisomes (2%), rough-

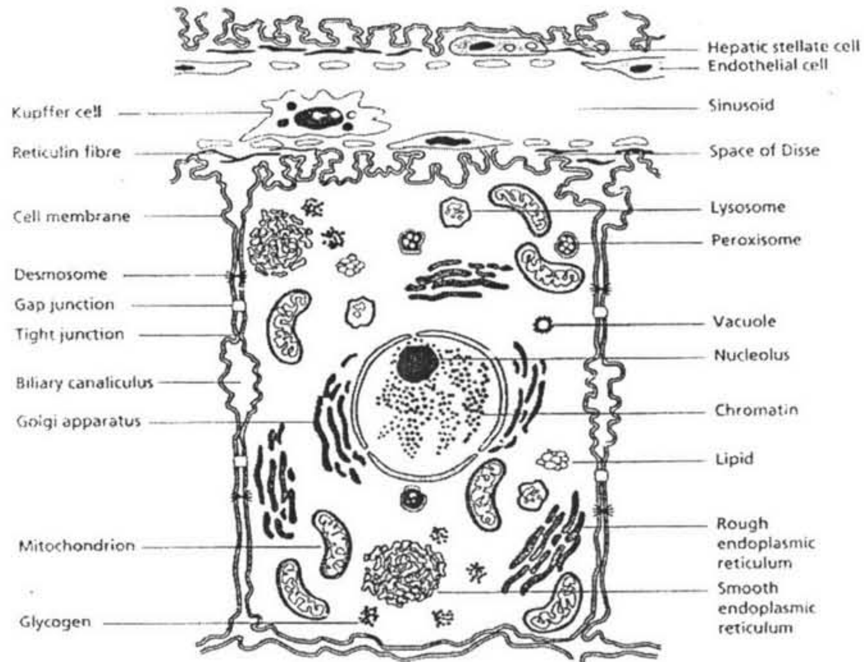


Figure 2. The organelles of the liver cell (Sherlock and Dooley, 2002).

endoplasmic reticulum (RER), smooth-endoplasmic reticulum (SER), lysosomes, golgi apparatus, granules of glycogen and lipid.

Furthermore, liver also contains non-parenchymal cells such as sinusoidal epithelial cells, kupffer cells, hepatic stellate cells (fat-storing cells or lipocytes or ito cells) and pit cells.

Liver receives blood supply at the site of the portal triad (portal tract) which contains the branch of hepatic portal vein, the hepatic artery and the bile duct (Figure 3). Blood enters via the portal vein (approximately 80%) carrying venous blood loaded with nutrients from the digestive viscera. The remaining 20% comes from the hepatic artery supplying oxygen-riched blood from the heart. Both vessels are mixed in the penetrating vessels, enters the sinusoids, percolates along the cords of hepatocytes, empties into central vein and exits the liver via the hepatic vein which join the inferior vena cava.

On the other hand, the bile flows in an opposite direction to that of the blood, bile flows from the bile canaliculi of zone 3 to zone 1 and enters to the bile duct in the portal tract (Junqueira and Carneiro, 1983).

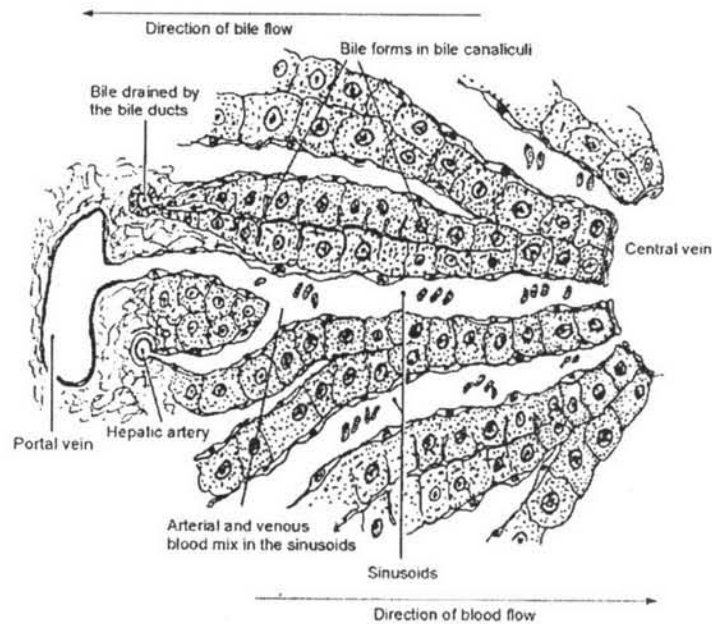


Figure 3. The direction of blood and bile flow in the liver.
(Ballantyne, Marrs, and Syversen, 2000).

The functional unit of the liver is the acinus. (Figure 4) The base of the acinus is formed by the terminal branches of the hepatic artery and portal vein branch from the portal tract. The acinus divides into 3 zones as follows:

Zone 1 called periportal area, cells are nearest the portal tract and receive well oxygenated blood and nutrient-riched blood, with a large of number of glutathione and mitochondria (Klaassen, 2001).

Zone 2 called midzonal area, intermediate area between zone 1and zone 3.

Zone 3 called periacinar or centrilobular area, cells are near the central vein and receive blood with the lowest concentrations of oxygen and nutrients, contain the largest quantities of cytochrome P450, especially CYP2E1, and the amount of glutathione less than zone 1 and 2 (Smith *et al.*, 1979), resulting that this area is more sensitive to injury.

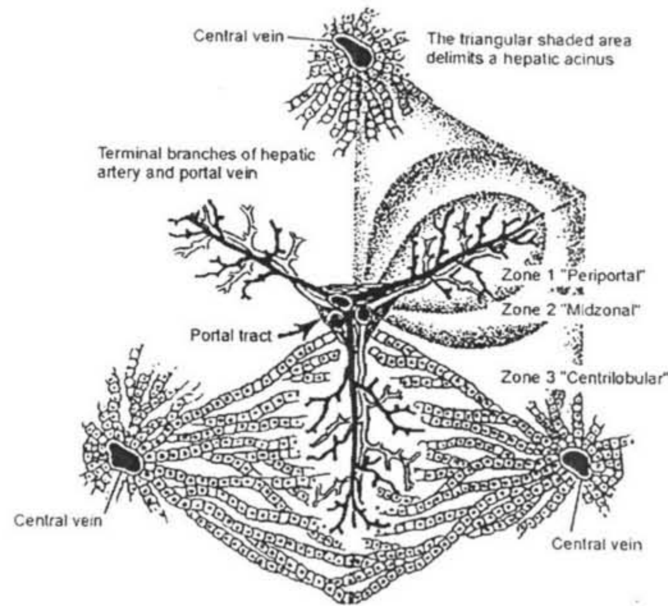


Figure 4. Diagram of the functional unit of the liver as acinus. (Ballantyne, Marrs, and Syversen, 2000).

Liver received most blood from the portal vein which may contribute to toxic agents exposure by oral route. In normal condition, each liver cell preserve homeostasis, but toxic agents exposure disturb normal homeostasis and may induce cellular adaptation. Cells were triggered to over cellular adaptation including metabolic disturbances and degeneration which are reversible. However, when cells are exposed to toxic agents consecutively until to point of no return, resulting in occurring irreversible cell injury is cell death which toxic agents can induce either apoptotic or necrotic cells depend on the characteristic of agents and the duration and extent of the insult. (วิจิตร บรรลุนารา, 2548)

Two different models responsible for liver cell death are apoptosis and necrosis (Figure 5) as follows:

Apoptosis, or programmed cell death, is a naturally occurring process of cell "suicide" that play a crucial role in the development and maintenance of body by eliminating aged, unwanted and damaged cells. It is energy-consuming process (Lee and Wei, 2000) which dying cells shrink and condense and then fragment, forming apoptotic bodies, which generally are phagocytosed by phagocytic cells with a lack of inflammation (Lodish *et al.*, 2000).

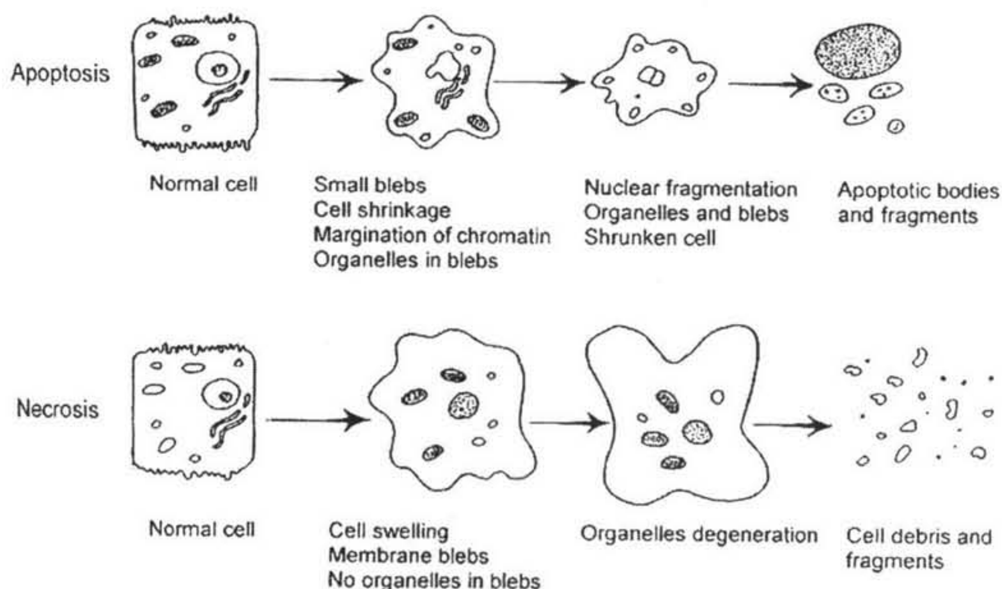


Figure 5. Morphology changes of apoptosis and necrosis (Zimmerman, 1999).

Necrosis, or accidental cell death, is process which occurring when cells are induced by a necrogenic agent and a serious physical insult. It unwanted energy to process which cell swelling, rupture of plasma membrane and damage to key organelles, releasing their intracellular contents, which can damage surrounding cells and cause inflammation (Boelsterli, 2003).

The liver has a remarkable capacity to regenerate after loss hepatic tissue due to the action of toxic agents or partial hepatectomy. Removal of 70% of the liver results in turning the livers from G_0 to S-phase with complete and rapid restoration of the liver size. The process of regeneration is probably stimulated and controlled by many substances including:

Chalones is substance acts as inhibitor of the mitotic cell division. When a tissue is injured or partially removed, the amount of chalones produce decreases, resulting in increasing of mitotic activity occurs in the liver (Junqueira and Carneiro, 1983).

Hepatocyte growth factor (HGF) and Transforming growth factoc-α (TGFα) are potent mitogens triggered cells to mitosis (Klaassen, 2001).

Epidermal growth factor (EGF) stimulates cells by increasing intracellular pH, Ca^{++}, polyamines and inositol triphosphate for the development of S-phase (Gitnick *et al.*, 1991).

Tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) promote proliferation of quiescent cells into cell cycle.

Furthermore, Noreepinephrine and Insulin potentiate the mitogenic activity for regeneration (Colorado State University, 1997).

On the other hand, the liver also has substance which involved in shutting down the regeneration that is Transforming growth factoc-β (TGF-β). It is a potent antimitogen and apoptogen which is the cessation of the liver regeneration (Klaassen, 2001).

Toxic agents are not only damage to normal liver cells but also damage to the liver regeneration, resulting in occurring of liver injury. It can directly interfere with the cellular and molecular mechanism of liver regeneration by damaging membranes (interruption of the interaction between growth factors and membranes receptors), impairment of gene expression and protein synthesis, stimulation of inflammatory reactions (increased production of cytokines and oxygen radicals) or activation of non-parenchymal cells (Palmes and Spiegel, 2004). The complete process of liver regeneration is one of the important treatment for many liver diseases, especially toxic agents induced liver injury.

2.3 Ethanol

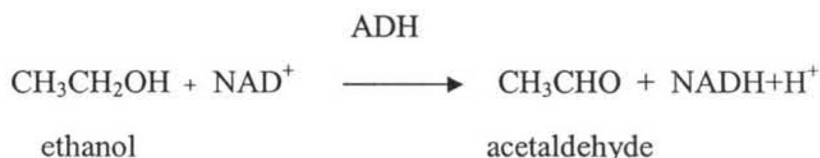
Ethanol (Alcohol or Ethyl alcohol) is a small, weakly polar and aliphatic hydrocarbon molecule that is both water and lipid soluble. The empirical formula is CH_3CH_2OH.

Absorption: Ethanol is rapidly absorbed from the gastrointestinal tract with in 30 to 60 minutes after ingestion. About 20 % absorbed in the stomach, the remainder (80%) absorbed in the small intestine. Factors that affect ethanol absorption are the amount and concentration of ethanol and gastric emptying time.

Distribution: Ethanol is both water and lipid soluble, so it distributes throughout body fluids and tissue depending on blood supply to various organs and tissues. Furthermore, ethanol can pass the blood brain barrier and placenta. Its mean volume of distribution (Vd) is 0.56 to 0.72 L/kg (Ford *et al.*, 2001).

Metabolism: Ethanol metabolism begins in the gastrointestinal cells by gastric mucosal alcohol dehydrogenase, but most of ethanol metabolism occur in liver with oxidation by 3 hepatic enzyme system as follows:

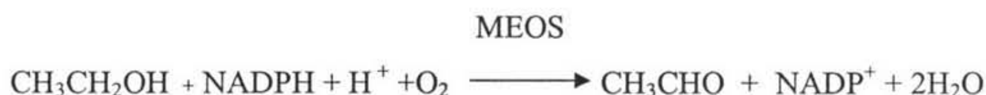
1.) Alcohol dehydrogenase (ADH): It is the main metabolic pathway of ethanol, even though at low ethanol levels (less than 0.05 %). This enzyme occurs mainly in the cytosol and catalyses the following chemical reaction:



$$CH_3CH_2OH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH{+}H^+$$

ethanol → acetaldehyde

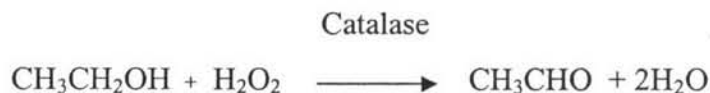
2.) Microsomal Ethanol Oxidizing System (MEOS): It is a usual metabolic pathway when blood ethanol is high or in case of chronic consumption of ethanol. This enzyme occurs mainly in the microsome and catalyses the following chemical reaction:



$$CH_3CH_2OH + NADPH + H^+ + O_2 \xrightarrow{MEOS} CH_3CHO + NADP^+ + 2H_2O$$

The main MEOS in this pathway is cytochrome P450-2E1 (CYP2E1), after chronic ethanol consumption, there is a 5-10 fold induction of CYP2E1 (Lieber, 2000)

3.) Catalase: It has only a minimal role in the metabolism of ethanol which occurs mainly in the liver peroxisome and catalyses the following chemical reaction:

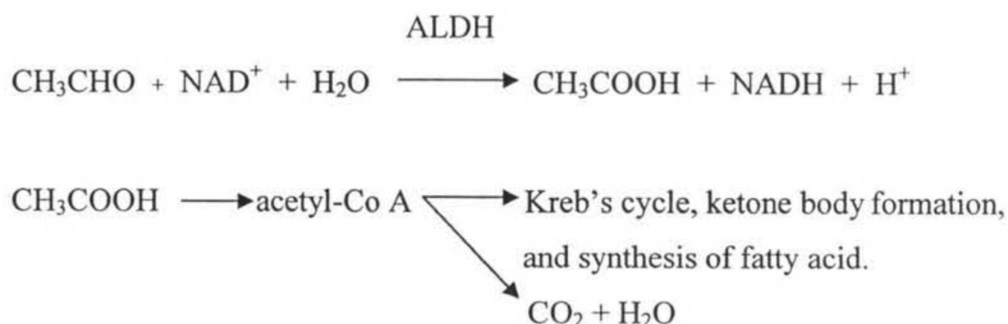


$$CH_3CH_2OH + H_2O_2 \xrightarrow{Catalase} CH_3CHO + 2H_2O$$

Furthermore, it is present in kidney and red blood cell which are the sites of extrahepatic ethanol oxidation.

Each metabolic pathway oxidizes ethanol to acetaldehyde which is a toxic metabolite, then mitochondrial aldehyde dehydogenase (ALDH) oxidizes acetaldehyde to acetate which is non-toxic metabolite. Acetate is converted to acetyl coenzyme A (acetyl-Co A) which then enters the Kreb's cycle, undergoes

ketone body formation, or is synthesized into fatty acid and oxidized to carbon dioxide (CO_2) and water (H_2O).



Individuals metabolize ethanol at rates of 100 to 200 mg/kg/hour or more, depending on previous exposure and genetic differences (Ford *et al.*, 2001).

Excretion: Most of ethanol is oxidized and excreted as CO_2 and H_2O. About 10% is excreted unchanged by urine, breath and sweat.

2.3.1 Ethanol induced liver injury

High dose or chronic ethanol consumption cause liver injury called alcoholic liver disease (ALD). There are 3 patterns of ALD as follows:

1.) Fatty liver or Steatosis is the accumulation of fat in the cytosol of hepatocytes primarily triglyceride for more than 5% of liver weight. It is chief importance as the first histologic evidence of adverse effect of ethanol on the liver. Most of the cells show large vacuoles that displace the nucleus. The more fat in the liver will affect the susceptibility to more severe liver damage (Jarvelainen, 2000). Fatty liver is a result of metabolic disturbances, such as decreased fatty acid oxidation, increased triglyceride synthesis, reduced fat export and mobilization of extrahepatic fat stores (Jarvelainen, 2000) and increased fatty acid synthesis (Zimmerman, 1999).

2.) Alcoholic hepatitis is the liver inflammation induced by alcohol characterized histologically by focal or larger areas of necrosis, aggregates of neutrophils, ballooning degeneration of hepatocytes, magamitochondria and alcoholic hyaline (mallory body or Mallory's hyaline) (Bouneva, *et al.*, 2003; Zimmerman, 1999). Centrilobular and perisinusiodal fibrosis may be occurred by proliferation of hepatic stellate cells (HSC). Their transformation into

myofibroblasts are the source of both the overproduction of structural proteins (constitute liver fibrosis) and matrix metalloproteinases (contribution in the remodeling of the hepatocytes) (Jarvelainen, 2000). The persistence of alcoholic hepatitis is associated with the progression of cirrhosis.

3.) Cirrhosis is an end-stage of ALD characterized by extensive liver fibrosis, micronodular regeneration, impaired liver function, portal hypertension and predisposition to hepatocellular carcinoma. Most death of ALD caused by the complication of cirrhosis, such as ascites, spontaneous bacterial peritonitis, variceal hemorrhage, hepatorenal syndrome and encephalopathy (วิญญู มิตรานันท์, 2538; Bouneva *et al.*, 2003).

2.3.2 Mechanism of ethanol induced liver injury

Ethanol caused pathogenetic effect on several ways as the result of ethanol itself, metabolites of ethanol oxidation and endotoxin activated kupffer cells.

1.) Ethanol itself caused liver injury

Ethanol is one of toxic chemicals, it can damage several cells and tissues with toxicity by itself.

2.) Metabolites and by-products of ethanol oxidation caused liver injury

2.1) Acetaldehyde is reactive and electrophilic metabolite which is the product of ethanol oxidation by alcohol dehydrogenase, MEOS and catalase. It is potentially a highly reactive substance that has direct cytotoxicity and an ability to from protein adduct with several protein of hepatocytes, such as tubulin, actin, calmodulin collagen and several enzymes etc., resulting in interference of lipoprotein secretion and microtubule formation, inhibition of DNA repair, impaired utilization of oxygen, enzyme inactivation, glutathione depletion, increased collagen synthesis, mitochondria toxicity by impairment of electron transport chain and decreasing oxidation of fatty acid and acetaldehyde including cause peroxidation of membrane lipids (Bouneva, 2003; Lieber, 2000a, 2000b; Zimmerman, 1999).

Furthermore, the covalent binding of adduct that act as neoantigens triggers the immunological response which consist primarily of class A immunoglobulin (IgA) and to some extent also from class G immunoglobulin (IgG) (Niemela, 2001).

Besides acetaldehyde can bind covalently to proteins, its and malondialdehyde (the aldehydic products of lipid peroxidation) react together with proteins in a synergistic manner and from hybrid protein adducts as malondialdehyde-acetaldehyde-protein adduct (MAA adduct). MAA adduct can stimulates antibodies against itself and also induced a proinflammatory response in hepatic endothelial cells by stimulating the secretion of several cytokines and chemokines, including TNFα, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) and induces profibrogenic response (Toma, 2002).

Therefore, both effect of acetaldehyde itself and several adduct types can lead to liver injury.

2.2) NADH (Nicotinamide adenine dinucleotide, reduced from)

Oxidation of ethanol to acetaldehyde by ADH,and oxidation of acetaldehyde to acetate by ALDH. Both reactions reduce nicotinamide adenine dinucleotide (NAD) to its reduced from NADH. The increased NADH/NAD ratio causes a number of metabolic disorders, including enhancing of triglyceride formation by increasing the concentration of α-glycerophosphate and trapping with fatty acid (Zimmerman, 1999), increasing of fatty acid synthesis (Lieber, 2000a) and inhibition of mitochondrial β-oxidation of fatty acid due to NAD depletion (Eaton *et al.*, 1997). Therefore, excess NADH can be attributed to the accumulation of fat and hyperlipidemia.

2.3) Free radicals

Free radicals such as α-hydroxyethyl radical, hydroxyl radical ($OH^{\cdot}$), and superoxide anion ($O_2^{\cdot}$) were generated by ethanol induced activity of CYP2E1, NADPH cytochrome P450 reductase and by the mitochondrial respiratory chain. Excess of free radicals leads to oxidative stress which may cause liver injury directly by initiating a chain of peroxidation or potentially activate inflammatory response by stimutation of the transcription of proinflammatory cytokines (Arteel, 2003; Papas, 1999; Jarvelainen, 2000; Zimmerman, 1999).

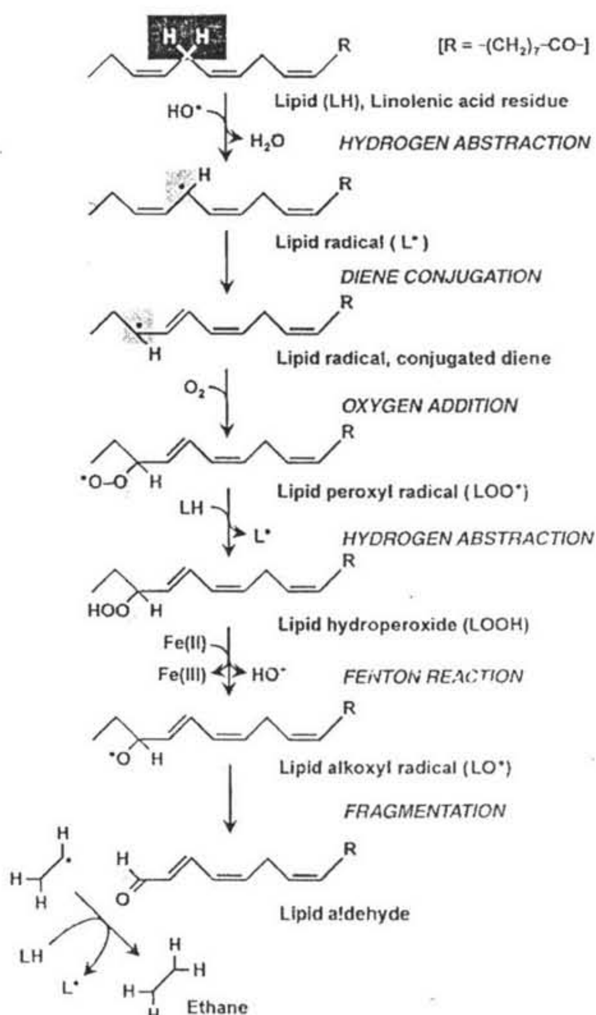


Figure 6. Mechanism of lipid peroxidation (Klassen, 2001).

Free radicals can initiate peroxidation of polyunsaturated lipids (Figure 6) by hydrogen abstraction from the hydrogen atoms on methylene carbons of unsaturated fatty acid which yields a lipid radical (L˙). Lipid radical abstracts methylene hydrogen from a neighboring lipid to generate a second lipid radical. These lipid radicals are unstable and undergone a series of transformations, including rearrangement of double bonds to give conjugated dienes. Lipid radicals react rapidly with O_2 to form lipid peroxyl radicals (LOO˙), then coverted to lipid hydroperoxide (LOOH) by hydrogen abstraction, and lipid alkoxyl radical (LO˙) by the Fe (II)-catalyzed Fenton reaction. Subsequent fragmentation gives rise to ethane and reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (Klassen, 2001; Hodgson and Smart, 2001).

Lipid peroxidation of plasma membrane and organelle such as mitochondria, lysosomes, rough and smooth endoplasmic reticulum contributed to cellular necrosis (Zimmerman, 1999).

Furthermore, the excessive of reactive oxygen species (ROS) in mitochondria may cause apoptosis by induction of mitochondrial membrane permeability transition and release of cytochrome C (Lee and Wei, 2000), and caspase activation (Adachi and Ishii, 2002).

3.) Activation of Kupffer cells caused liver injury

Ethanol can increase intestinal permeability by disruption of both actin and microtubule cytoskeletons that regulates paracellular permeability (Keshavarzian *et al.*, 2001), resulting in the increased passing of Lipopolysaccharide (LPS) or endotoxin which derives from the outer cell wall material of gram-negative bacteria to the portal vein. Excess LPS activated kupffer cells to produce proinflammatory cytokines such as TNFα and IL-1β, prostaglandins (particularly PGE_2), free radicals both reactive oxygen species (ROS) sush as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and reactive nitrogen species (RNS) such as nitric oxide (NO^-) (Mckim *et al.*, 2003), and chemokines for the recruitment of polymorphonuclear leukocytes (Neutrophils) to the liver (Nagy *et al.*, 2003). A variety of substances form the activation of kupffer cells contribute to the progression of liver injury.

2.3.3 The parameters indicated ethanol induced liver injury

Clinical chemistry parameters together with histopathological examination can be used to assess biological changes leading to the prediction of disease, disease states and progression of disease induced by ethanol. The important parameters to indicate liver injury by ethanol are as follows:

1.) Clinical chemistry parameters

1.1) Aminotranferases

Aminotranferases are wildly used both experimentally and clinically as markers for liver injury induced by several toxic agents (Boelsterli, 2003), including ethanol (Bouneva *et al.*, 2003).The important enzymes are alanine aminotransferase (ALT; also called serum glutamate pyruvate transaminase, SGPT) and aspartate aminotransferase (AST; also called serum glutamate oxaloacetate transaminase,

SGOT). Both enzymes are a group of enzymes which catalyses the reversible transformation of a keto acids into amino acids by transfering of amino groups (บุญมี สัญญสุจจารี, 2544). ALT is present in a large quantities in the cytosol of hepatocytes and less ALT in heart and skeleton muscles. While AST is presented in the mitochondria of liver, heart, skeleton muscle and kidney (Sherlock and Dooley, 2002). In normal physiological condition both enzymes are in hepatocytes. In contrast, necrosis or deterioration in integrity of the hepatocyte plasma membrane from toxic agents can lead to discharge of ALT and AST into the serum (Hodgson and Smart, 2001) which elevation more than normal values. Serum increase in ALT is more specific for liver injury than AST.

1.2) Triglycerides

Triglycerides are simple lipids which occurred from the esterification of glycerol with three fatty acids. It acts as an important energy source forming much of body fat storage and also a method of energy transport from the gut and liver to peripheral tissues (Sherlock and Dooley, 2002). Normal hepatocytes have a small amount of fat vacuoles containing triglycerides. However, Consumption of ethanol produced increasing of fat accumulation by increasing triglyceride and fatty acid synthesis, decreasing fatty acid oxidation, increasing delivery rate of fatty acid and mobilization of lipid from peripheral depots to the liver, and decreasing lipoproteins to the blood (Zimmerman, 1999). Therefore, the amount of triglyceride may be used as an indicator of ethanol induced fatty liver (Kwon, Kim and Choung, 2004; Siler *et al.*, 1998; Poggi and Di Luzio, 1964).

1.3) Glutathione

glutathione is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) consisting of glutamate, cysteine and glycine which is synthesized in the cytosol and distributed in intracellular organelles, including endoplasmic reticulum and mitochondria of all mammalian cells. It is important for antioxidant defense, regulation of cell cycle and gene expression (Dicknson and Forman, 2002). More than 98% of intracellular glutathione is reduced glutathione (GSH) (Deleve and Kaplowitz, 1991). GSH is most abundant in hepatocytes about 4-8 mM. (Hodgson and Smart, 2001) and is one of the most important defense molecules in the liver which has a reactive sulfhydryl (-SH) group for its antioxidant activities. The role of GSH in this activity includes a free radical scavenger, a co-substrate for GSH-related enzyme (glutathione

peroxidase), a regenerator of α-tocopherol and plays an important role in the maintenance of non-protein sulfhydryl groups in the cell (Song *et al.*, 2006). Decreasing the amount of GSH by toxic agents contributes to oxidative stress that can cause lipid peroxidation and resulting in cell injury.

1.4) Malondialdehyde

Malondialdehyde is an end-product of lipid peroxidation. Ethanol consumption induced increasing generation of free radicals together with reduction of liver antioxidant causing oxidative stress and leading to lipid peroxidation. Excess lipid peroxidation can be measured by formation of malondialdehyde (MDA). Therefore, the amount of MDA may act as an indicator of liver injury causing by oxidative stress (Teare *et al.*, 1994; Saravanan, Visvanathan and Pugalendi, 2006).

1.5) Cytokines

Cytokines are peptide which have the role as primary chemical messengers involved in both acute and chronic inflammation, attracting and activating phagocytes, promoting the clotting of blood, and facilitating the production of additional chemical messengers, including more cytokines (McClain *et al.*, 1997).

Ethanol consumption induced elevation of endotoxin pass to the liver. These endotoxin stimulated kupffer cells to produce proinflammatory cytokines such as TNFα and IL-1β, the two important mediators of inflammation and cell death (Jarvellainen *et al.*, 1999; Boelsterli, 2003). Therefore, the amount of TNFα and IL-1β may act as an indicator of liver injury induced by ethanol (Bautista and Spitzer, 1999; Tilg and Diehl, 2000).

2.) Histopathological parameters

To confirm the result of ethanol induced hepatic disorders and the effective protection and treatment of plant extract. The most common method is histopathological examination, performed by tissue section, standard and special staining, microscopic lesions were then investigated and analysis.

The histological changes that can be found in ethanol induced liver injury (Zimmerman, 1999) include cell swelling, large fat vacuoles, focal or large areas of necrosis, alcoholic hyaline, aggregation of neutrophils, fibrosis etc. In addition, those cells mentioned less accumulation of glycogen than normal cells.

2.4 Silymarin

Silymarin is a flavonoid extracted from the fruits and seeds of the milk thistle, *Silybum marianum*. It contains flavonolignans such as silybin, silydianin and silychristine. Various studies also indicate that silymarin exhibits anti-carcinogenic effect, anti-inflammatory effect, antioxidant effect and protective effect against hepatotoxicity induced by a variety of toxic agents including ethanol both in animal models and human clinical trails (Flora *et al.*, 1998; Kang *et al.*, 2004; Song *et al.*, 2006). Therefore, this study used silymarin as a positive control.