CHAPTER II

LITERATURE REVIEWS

Hibiscus sabdariffa Linn.

H. sabdariffa is a medicinal plant belongs to family Malvaceae. It is natively grown in tropical Africa but nowadays it is grow throughout many tropical climate regions. It has been called differently in different locations such as Jamaica roselle (in Mexico), Karkade roselle (in Switzerland), Roselle or Jamican roselle (in England). It is widely cultivated in Egypt, Sudan, Thailand. It has been known in Thailand as Krachiap, Krachiap-daeng, Krachiap-prieo (Central), Phak-Kheng-Kheng (North), Som-poo (Mae-Hong Son), Som-por-dee (Northeast) (Boonyapraphastsara, N., 1987; Farnsworth, N.R., and Bunyapraphatsara, N., 1992; Marderosian, A.D., and Beutler, J.A., 2002).

In Thailand, *H. sabdariffa* is used as a traditional medicine as diuretic, hypocholesterolemic agent, antihypertensive agent, mucolytic agent, intestinal peristalsis stimulation, bile acid secretion enhancement, mild laxative and as refreshing beverages (พร้อมจิต ศรลัมพ์, 2532; สุนทรี สิงหบุตรา, 2540; Boonyapraphastsara, N., 1987).

Various parts of this plant have been used in traditional medicine as following (Farnsworth, N.R., and Bunyapraphatsara, N., 1992) :

1. Leaves: for treatment of trichinosis, cough and as an expectorant.
2. Flowers: for treatment of kidney stones, urinary bladder stones and strangury, hypercholesterolemia and as an expectorant.
3. Fruits: for alleviation of thirst, treatment of gastric ulcers and hypercholesterolemia.
4. Seeds: for health promotion, treatment of biliary disease, hypercholesterolemia and as a diuretic and element tonic.
5. Not specified parts: for fever, alleviation of thirst, health promotion, treatment of fatigue, biliary disease, disorders of urination, cough, as an antihypertensive, antihypercholesterolemia, expectorant, blood tonic, element tonic, antidiabetic, and vasodilator.

Botanical description of *H. sabdariffa* (Figure 1)

H. sabdariffa is an erect annual herb with reddish, cylindrical stem, nearly or quite glabrous. Leaves are simple, having petiole, blade 3-5 lobed or parted, the lobes serrate or obtusely toothed. Flowers are solitary, axillary, nearly sessile, 5-7 centimetre in diameter; consisting of 8-12 epicalyx-segments, distinct, lanceolate to linear, adnate at base of the calyx; thick calyx, red, and freshy, cup-like, deeply parted, prominently 10-nerved; 5 petals, yellow, twice as long as calyx; numerous stamens, the filaments united into a staminal column; single style, 5-branched near summit, stigma capitate. Fruit is capsule, ovoid, pointed, 1-2 centimetre long, shorter than the calyx, having densely sharp and stiff hairs, loculicidally dehiscent. It is propagated by seeds. Seeds are sown at the beginning of the rainy season, and the seedlings, when ready, are planted out at about 4 x 3 feet spacing. The crop should be harvested in 5-6 month after planting (Farnsworth, N.R., and Bunyapraphatsara, N., 1992).

Figure 1 The calyx of *Hibiscus sabdariffa* Linn.

Chemical constituents found in the calyx of *H. sabdariffa*

These following constituents were found in the calyx of *H. sabdariffa* :

1. **Organic acids:** citric acid, malic acid, tartaric acid, and (+)-allo-hydroxycitric acid lactone (hibiscus acid) (Miller, L.P., 1973)

Citric acid

Citric acid is a hydroxyl tricarboxylic acid. This polybasic acid is abundant in fruit juices, widely distributed in fungi and in higher plants, occurring in the free state in various plant extracts with comparatively high concentration.

Biologically, the major pathway of citric acid synthesis is through the formation of a C-C bond between oxaloacetate and acetyl-CoA under the action of citrate synthetase, also known as citrate condensing enzyme.

Malic acid

Malic acid contains an asymmetric carbon center and is therefore known in two active forms. The naturally occurring malic acid is the L(-)-malic acid. L(-)-malic acid, in general is the principal organic acid in a great number of plants. It is universally distributed in living organisms. The richest sources are apple, tamarinds, rhubarb, tobacco leaves, and *Kalanchoe* leaves.

L(-)-malic acid is formed from the hydration of fumaric acid. This reaction is reversibly catalyzed by a soluble enzyme, fumarate hydratase (or fumarase), which is specific for these substrates.

Tartaric acid

Tartaric acid exists in several forms: L-tartaric acid, D-tartaric acid, and *meso*-tartaric acid. The naturally occurring acid has a levo configuration. This acid is widely distributed in plants, especially in tamarinds and other fruits, in the free state and as its salt of potassium or calcium. The tartaric acid isolated from *Bauhinia* has been reported to be the D-isomer.

Tartaric acid is somewhat similar to oxalic acid in respect to its metabolic inertness in certain plants. Although the presence of both oxalic and tartaric acids in plant tissues has been long reported, the metabolic transformation of these acids has not been elucidated.

<u>(+)-Allo-hydroxycitric acid lactone (Hibiscus acid)</u>

(+)-Allo-hydroxycitric acid lactone (hibiscus acid) is hydroxycitric acid derivatives, which is a unique hydroxycitric acid that can find in *H. sabdariffa*. Hydroxycitric acid (HCA) is the principal acid found in *Garcinia cambogia*, *Garcinia indica*, *Garcinia atroviridis*, *H. sabdariffa*, beet sugar. It is discussed with reference to its use as a metabolic regulator of obesity an its potential for application as an ingredient in functional foods (Fletcher, R., 1997) and HCA is susceptible to lactonisation especially during evaporation and concentration (Jayaprakasha, G.K., and Sakariah, K.K., 2002).

2. Flavonoids: gossypetin, quercetin, anthocyanins (cyanidin, delphinidin, hibiscin)

Flavonoids are 3-ring phenolic compounds consisting of a double ring attached by a single bond to the third ring. The flavonoids are found in fruits, vegetables, nuts, seeds, herbs, spices, stems, flower, as well as tea and red wine. They are prominent components of citrus fruits. Flavonoids are effective in antiinflammation, antiplatelet, allergic disease, coronary artery disease, breast cancer, protect cells against oxidative stress (Ebadi, M., 2002; Mills, S., and Bone, K., 2000; Rice-Evans, C.A., 2003).

The flavonoids that found in *H. sabdariffa* include gossypetin, quercetin, anthocyanins. Anthocyanins are water soluble flavonoids that present the colour properties; thus cyanidin is crimson and delphinidin is mauve. In addition, there is another anthocyanin also found in *H. sabdariffa* such as hibiscin (Delphinidin-3-xylosylglucoside) (Miller, L.P., 1973; Phillipson, J.D., 1994).

3. Phenolic compounds (Phenolic acid): protocatechuic acid (PCA) or 3,4-dihydroxybenzoic acid

Phenolic compounds composed of one or more aromatic benzene rings with one or more hydroxyl groups (C-OH). PCA is phenolic acid that can find in *H. sabdariffa*. Furthermore, PCA is also found in a number of soft fruits and vegetables in the form of glucosides, such as in grape, strawberry, black berry, black currants. PCA is shown to be able to inhibit LDL lipid peroxidation and to reduce the extent of its cytotoxic activity (Rice-Evans, C.A., 2003).

Chemical structures of these constituents in *H. sabdariffa* were shown in Figure 2.

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Organic acid: citric acid

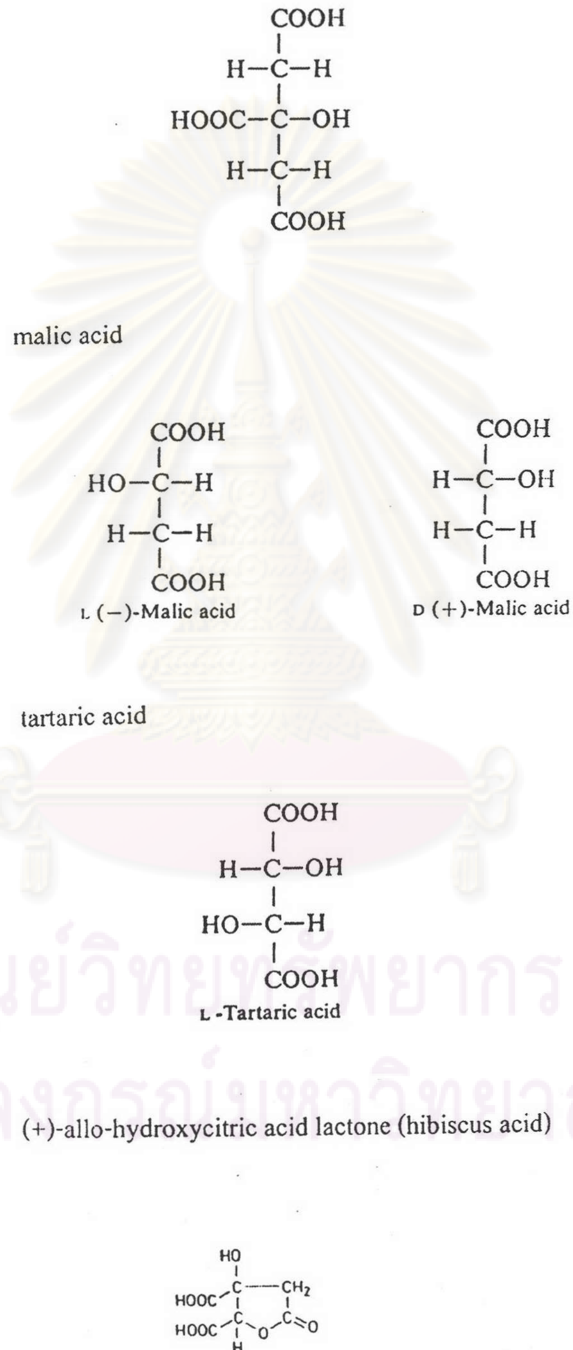

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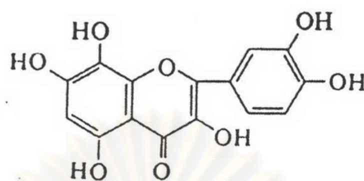
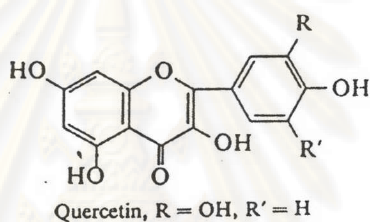
Figure 2 Chemical constituents found in *H. sabdariffa* (Miller, L.P., 1973; Phillipson, J.D., 1994)

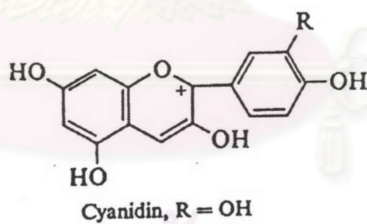
Flavonoids: gossypetin



quercetin



cyanidin



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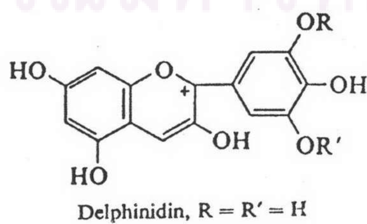
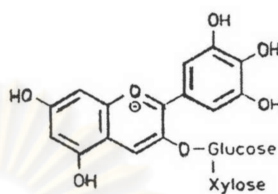


Figure 2 *(continued)* Chemical constituents found in *H. sabdariffa* (Miller, L.P., 1973; Phillipson, J.D., 1994)

hibiscin



Phenolic compounds: protocatechuic acid (PCA)

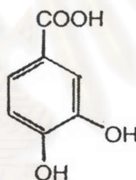


Figure 2 *(continued)* Chemical constituents found in *H. sabdariffa* (Miller, L.P., 1973; Phillipson, J.D., 1994)

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Pharmacological effects

Antihypertensive effect

An intravenous administration of the aqueous extract of *H. sabdariffa* at doses of 62.5, 125 and 250 mg/kg caused hypotension and bradycardia in normotensive rats. In isolated guinea-pig atria, the extract exhibited negative inotropic and chronotropic effects which were found to be concentration dependent. Additionally, *H. sabdariffa* showed vasorelaxant effect when tested on aortic ring with endothelium intact and endothelium denuded which precontracted by phenylephrine (Tiamjan, R., 1999). Roselle calyx extract infusion at doses of 500 and 1,000 mg/kg body weight was found to lower both systolic and diastolic blood pressure in spontaneous hypertensive and normotensive Wistar-Kyoto rats. Urine output of the treated spontaneous hypertensive rats was significantly higher than those of the control group. As compared to the control group, serum creatinine, cholesterol and glucose in the treated rat was decreased while serum uric acid was increased (Onyenekwe, P.C. *et al.*, 1999). Effect of aqueous extract of *H. sabdariffa* was studied in the established stage of 2-kidney, 1-clip renovascular hypertensive Sprague-Dawley rats. After 8 weeks of the administration of the extract at 250 mg/kg/day in drinking water, the systolic, diastolic blood pressure and heart rate were lower than those of the control hypertensive rats (Odigie, I.P., *et al.*, 2003). Effectiveness and tolerability of *H. sabdariffa* in patients with mild to moderate hypertension were investigated in patients of age between 30-80 years old with diagnosed hypertension and without antihypertensive treatment for at least 1 month before the experimentation. Patients in experimental treatment group received 10 g of *H. sabdariffa* dry calyx in 0.5 L. of water (contained 9.6 mg total anthocyanins contents) daily before breakfast, whereas patients in control treatment group received captopril 25 mg twice a day, for 4 weeks. The results showed that *H. sabdariffa* was able to decrease the systolic blood pressure from 139.05 to 123.73 mm.Hg and diastolic blood pressure from 90.81 to 79.52 mm.Hg. At the end of the study, there were no significant difference between the blood pressure detected in *H. sabdariffa* treatment group and captopril treatment group (Herrera-Arellano, A., *et al.*, 2004).

Hypo-cholesterolemic effect

Dried calyx extract of *H. sabdariffa* was administered at the doses of 500 mg/kg/day and 1,000 mg/kg/day for 6 weeks to male Sprague-Dawley rats which were induced to become hypercholesterolemia by continually feeding with cholesterol dissolved in corn oil. The results demonstrated that serum cholesterol, triglycerides and LDL-cholesterol level were decreased while serum HDL-cholesterol level was not affected by *H. sabdariffa* administration. In addition, administering the extract at 250, 500 and 1,000 mg/kg/day to the hypercholesterolemic rats for 6 weeks decreased TBARs formation by about 40% (Hirunpanich, V., 2001). Feeding New Zealand white rabbits with 0.5 and 1 % of *H. sabdariffa* extract in high cholesterol diet for 10 weeks resulted in low serum levels of triglycerides, total cholesterol and LDL-cholesterol as compared to the control rabbits fed only high cholesterol diet. The number of foam cells in atherosclerotic lesions in the thoracic aorta were shown to decrease in *H. sabdariffa* treatment groups (Chen, C.C., *et al.*, 2003).

Gastroprotective effect

Gastroprotective effect of the aqueous extract of *H. sabdariffa* was examined in rats. Oral administration of the extract from 250-1,000 mg/kg significantly inhibited gastric ulcer formation induced by indomethacin, ethanol and hypothermic restraint-stress (Rujjanawate, C., *et al.*, 2000). The *H. sabdariffa* mucilage fraction was isolated and examined in experimentally ulcerated rats. The results revealed that oral administration of mucilage from 125-500 mg/kg significantly inhibited gastric ulcer formation induced by indomethacin, ethanol and hypothermic restraint-stress (Rujjanawate, C., *et al.*, 2001).

Antioxidative effect

Hibiscus anthocyanins (HAs) were studied for antioxidant bioactivity using the model of *tert*-butyl hydroperoxide (*t*-BHP)-induced cytotoxicity in rat primary hepatocytes and hepatotoxicity in rats. The results demonstrated that HAs at the concentrations of 0.10 and 0.20 mg/ml significantly decreased the leakage of lactate dehydrogenase and the formation of malondialdehyde induced by a 30-min treatment of *t*-BHP (1.5 mM). The *in vivo* investigation showed that the oral pretreatment of HAs (100 and 200 mg/kg/day) for 5 days before a single

dose of *t*-BHP (0.2 mmol/kg; i.p.) significantly lowered the serum levels of hepatic enzyme markers (alanine and aspartate aminotransferase) and reduced oxidative liver damage. Histopathological evaluation of the liver revealed that *Hibiscus* pigments reduced the incidence of liver lesion including inflammatory leukocyte infiltration, and necrosis induced by *t*-BHP in rats (Wang, C.J., *et al.*, 2000). Antioxidative potential of three fraction of the ethanol crude extract (chloroform soluble fraction, ethyl acetate soluble fraction, residual fraction) obtained from the dried flowers of *H. sabdariffa* were evaluated for their capacity of quenching 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical and inhibiting xanthine oxidase (XO) activity. Ethyl acetate soluble fraction showed the greatest capacity of scavenging free radical (EC_{50} = 0.017 mg/ml) and chloroform soluble fraction showed the strongest inhibitory effect on XO activity (EC_{50} = 0.742 mg/ml). Further investigation was studied using a model of *t*-BHP-induced oxidative damage in rat primary hepatocytes. Exposure of isolated hepatocytes to *t*-BHP resulted in leakage of LDH and ALT, peroxidation of cellular lipids. All fractions were found to inhibit significantly the unscheduled DNA synthesis (UDS) induced by *t*-BHP at a concentration of 0.20 mg/ml. Chloroform soluble fraction and ethyl acetate soluble fraction also decreased the leakage of lactate dehydrogenase (LDH) and the formation of malondialdehyde (MDA) induced by *t*-BHP (1.5 mM) considerably at a concentration of 0.10 and 0.20 mg/ml in the rat primary hepatocyte cultures (Tseng, T.H., *et al.*, 1997). *Hibiscus* protocatechuic acid (PCA) is a simple phenolic compound isolated from calyx of *H. sabdariffa*. After pretreatment with PCA for 10 min at the final concentration of 0.02, 0.05 or 0.10 mg/ml, hepatocytes were incubated with *t*-BHP (1.5 mM) for 30 min. PCA at concentrations of 0.05 and 0.10 mg/ml significantly decreased the leakage of LDH, ALT and the formation of MDA (Tseng, T.H., *et al.*, 1996). The investigation of PCA on antioxidative and anti-inflammatory effects *in vivo* showed that pretreatment with PCA (50-100 mg/kg) by gavage for 5 days before a single dose of *t*-BHP (0.2 mM/kg; i.p.) significantly lowered serum levels of the hepatic enzyme markers; LDH, ALT and AST, as well as reduced oxidative stress of the liver , as shown by evaluating of MDA and glutathione (GSH). Histopathological evaluation of the rat livers revealed that PCA reduced the incidence of liver lesions including hepatocyte swelling, leukocyte infiltration, and necrosis induced by *t*-BHP (Liu, C.L., *et al.*, 2002). In a study, using lipopolysaccharide (LPS, an endotoxin from *Escherichia coli*) to induce rat liver inducible nitric oxide synthase (iNOS) and

hepatic damage, showed that pretreatment of rats with PCA (0.2 and 0.5 mmol/kg/day by gavage) for 5 days significantly decreased serum levels of the hepatic enzyme markers (ALT, AST) induced by the 6 hours treatment with LPS (5 mg/kg; i.p.). Furthermore, PCA reduced the incidence of liver lesions induced by LPS, including neutrophil infiltration, congestion, and liver cells swelling (Lin, W.L., *et al.*, 2003).

Antimutagenic effect

The 80% ethanol extract of roselle reduced about 60-90% of the mutagenicity induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine (PhIP) and other heterocyclic amines at a concentration of 12.5 mg/plate in the *Salmonella* mutation assay. The inhibitory effects of the extract in F344 rats in which aberrant crypt focus (ACF) formation was induced by azoxymethane (AOM) and PhIP. In the initiation stage of carcinogenesis, the extract at the dosage of 1.0 g/kg body weight decreased number of AOM-induced ACF in the colon for 17-25 % and number of PhIP-induced ACF in the colon for 22 % (Chewonarin, T., *et al.*, 1999). *Hibiscus* protocatechuic acid (PCA) was evaluated for its ability to inhibit the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced promotion in skin tumor of female CD-1 mice. Topical applications of PCA (5, 10 or 20 μmol) to mice 5 min prior to TPA (15 nmol) treatment twice weekly for 20 weeks (which were tumor-initiated with benzo[*a*]pyrene) were found to inhibit the incidence of tumors in mice for 81.3, 62.5 and 56.3%, respectively, while all mice in the TPA-treated group developed tumors. The protective effects of PCA were also presented by its significant suppression of the TPA-induced hyperplasia in the skin and edema of mouse ears by 65% and 73% at doses of 10 and 20 μmol, respectively. When it was applied to the dorsal surface of CD-1 mice before TPA application, PCA (5, 10 or 20 μmol) inhibited the activity of epidermal ornithine decarboxylase (ODC) enzyme induced by 5 nmol of TPA and of myeloperoxidase (MPO) enzyme induced by 6.5 nmol of TPA. PCA (5, 10 or 20 μmol) also inhibited the formation of hydrogen peroxide in the mouse skin by 61, 84 and 89%, respectively, as compared to the TPA-treated group (Tseng, T.H., *et al.*, 1998).

Neuropharmacological effect

The neuropharmacological effects of the aqueous extract of *H. sabdariffa* were studies in rodent. The extract at doses of 100, 200 and 400 mg/kg, i.p. caused a remarkable dose

dependent decrease in spontaneous motor activity in mices, increased the duration of pentobarbital (40 mg/kg; i.p.)-induced sleep in rats and reduced the exploratory behaviour in mice. The extract significantly inhibited the intensity of apomorphine (1 mg/kg; s.c.)-induced stereotypic behaviour and attenuated climbing of the mice in a dose dependent manner (Amos, S., *et al.*, 2003).

Cathartic activity

The cathartic activity of lyophilized aqueous extract of roselle 800 mg/kg was investigated. It was shown to cause a pronounced increase in the amount of wet faeces in rats without any significant increase in the propulsive intestinal movement. Unlike senna (the positive control), normal defecation was not affected as compared to the distilled water treated group (the negative control). The cathartic property of the extract may be due to the saponin-like compounds contained in the extract (Haruna, A.K., 1997).

Toxicological effects

Clinical study

Thirty six healthy male medical students aged between 20-30 year old participated in the study. They were allowed to drink roselle extract at 16 or 24 g/day for 7 days. The results showed that their urinary pH were unchanged whereas creatinine, uric acid, citrate, tartrate, calcium, sodium, potassium and phosphate are decreased but no changes of oxalate in the urinary excretion (Kirdpon, S., *et al.*, 1994).

Toxicological investigation

Dried powder of *H. sabdariffa* was extract with the mixture solvent of methanol and water (4:1). The extract was given orally to Wistar albino rats. The rats were divided into 6 groups in which rats received the extract for 0, 1, 3, 5, 10 and 15 doses of 250 mg/kg, respectively. The results showed that serum AST and ALT were significantly increased in all of the *H. sabdariffa* treatments as compared to the control group receiving 0.9% NaCl. Serum levels of alkaline phosphatase, and lactate dehydrogenase were not significantly affected. Only the group receiving 15 doses of the extract possessed a significant increase of serum albumin.

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However, the histopathological evaluation revealed no pathological changes in livers and hearts in all of the *H. sabdariffa* treated groups (Akindahunsi, A.A., and Olaley, M.T., 2003).

Acute and subchronic toxicity studies

The median lethal dose (LD_{50}) was found to be 5 g/kg body weight. *H. sabdariffa* aqueous extract was given to rats at doses of 1.15, 2.30, 4.60 g/kg in drinking water for 12 weeks. The results showed that significant reduction in food and water consumption were observed in the treated groups as compared to the control group. Serum levels of potassium, sodium, chloride and bicarbonate were significant decreased while serum levels of BUN and creatinine were significant increased. Body weight and weight of the kidneys were significant decreased. The major histopathological feature was tubular necrosis with areas of degeneration of which severity was dose-dependent (Orisakwe, O.E., *et al.*, 2003). Subchronic effect of *H. sabdariffa* aqueous extract on rat testes was investigated with three tested groups receiving the extract dissolving in drinking water at doses of 1.15, 2.30 and 4.60 g/kg for 12 weeks. The results showed that a significant decrease of epididymal sperm counts was observed in the 4.60 g/kg group. Rats in the 2.30 g/kg group showed hyperplasia of testis with thickening of the basement membrane, while rats in the 1.15 g/kg group showed distortion of tubules and a disruption of normal epithelial organization as compared to the control (Orisakwe, O.E., *et al.*, 2004).

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Metabolic bioactivation of xenobiotics

Xenobiotics are foreign chemical compounds to the body, such as drugs, food additives, and environmental pollutants (Murray, R.K., 1993). Mammals are equipped with a variety of enzyme systems that catalyze the transformation of xenobiotics to form, in general, more polar metabolites that are more readily excreted in urine or feces and that are less likely to have access to and to interact with membrane-bound receptors (Woolf, T.F., 1999).

Xenobiotic metabolism is a biological process which alters many substances leading to termination or alteration of biological activity. Xenobiotic metabolizing enzymes occur in many organs. The organ most commonly involved in the biotransformation of foreign compounds is liver because of its position, blood supply and function. Most foreign compounds are taken into the organism via the gastrointestinal tract, and the blood that drains the tract flows through the portal vein directly to the liver. Therefore liver represents a portal to the tissues of the body and is exposed to foreign compounds at higher concentrations than most other tissues. Detoxication in this organ and possible removal by excretion into the bile are therefore protective measures. The role of liver in endogenous metabolism and its structure make it an ideal site for the biotransformation of xenobiotics. Metabolism in the liver may be so extensive during the " first pass " of the compound through the organ that little or none of the parent compound reaches the systemic circulation. Other organs that display this function include gastrointestinal tract, lungs, skin, and kidneys. Enzymes are found in smooth endoplasmic reticulum (SER). Some are located in the cytosol and a few are found in other organelles such as the mitochondria.

In general, metabolism can be simply and conveniently divided into two phases: phase I and phase II. Phase I is the alteration of the original foreign molecule so as to add on a functional group which can then be conjugated in phase II. Most biotransformations can be divided into phase I and phaseII reactions, although the products of phase II biotransformations may be further metabolized in what is sometimes termed phase III reactions. If the foreign molecules already possesses a functional group suitable for a phase II reaction, a phase I reaction will be unnecessary (Timbrell, J., 2000).

Table 1 The major biotransformation reactions (Timbrell, J., 2000)

Phase I	Phase II	Phase III
Oxidation	Sulphation	Further metabolism of
Reduction	Glucuronidation	Glutathione conjugates
Hydrolysis	Glutathione conjugation	
Hydration	Acetylation	
Dehalogenation	Amino acid conjugation	
	Methylation	

Phase I reactions

Phase I reactions includes oxidation, reduction, hydrolysis and hydration in order to reveal or introduce functional or reactive groups such as OH, SH, NH_2, CO_2H in the molecules (Ioannides, C., 1996; Woolf, T.F., 1999). The reactions will be discussed in terms of reaction type and, with respect to oxidation, site of enzyme. Oxidation performed by the microsomal mixed-function oxidase system (cytochrome P450-dependent) is considered separately because of its importance and the diversity of reactions performed by this enzyme system (Gibson, G.G., and Skett, P., 1994).

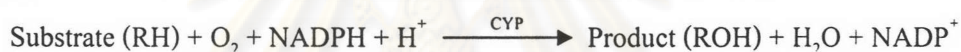
Cytochrome P-450 mono-oxygenase system

The most important enzyme system catalyzing phase I metabolic reaction is the cytochrome P-450 mono-oxygenase system. In 1958, it was discovered that when pig or rat liver microsomes were treated with dithionite and purged with carbon monoxide, a strong absorption band occurred at 450 nm that was most unusual for the known pigments. The pigment responsible for this phenomenon was called P (for pigment) 450 (Ioannides, C., 1996).

The P450 enzymes are hemoproteins with approximate molecular weights of 50,000 Da. These enzymes catalyze the monooxidation of a wide variety of structurally unrelated compounds, including endogenous steroids and fatty acids and essentially unlimited number of lipophilic xenobiotics. Those members of this family of enzymes that participate in steroidogenesis and found in the mitochondria and endoplasmic reticulum of steroidogenic

organs (Woolf, T.F., 1999). Cytochromes P-450 mono-oxygenase (CYPs) is located particularly in the SER of the cell. The enzyme system is isolated in the microsomal fraction which is formed from the endoplasmic reticulum when the cell is homogenized and fractionated by differential ultracentrifugation. Microsomal vesicles are thus fragments of the endoplasmic reticulum in which most of the enzyme activity is retained (Potter, T.D., and Coon, M.J., 1991).

The reactions catalyzed by the P450s involve the four-electron reduction of dioxygen, which is coupled to the two-electron oxidation of a substrate (RH) and the two-electron oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to form the oxidized product (ROH), $NADP^+$ and water. Enzymatic activity is dependent on a flavoprotein, NADPH-cytochrome P450 reductase, that passes reducing equivalents from NADPH to the cytochrome P450 substrate complex. The basic reaction catalyzed by CYP is a monooxygenation reaction as following (Gibson, G.G., and Skett, P., 1994):



$$\text{Substrate (RH)} + O_2 + \text{NADPH} + H^+ \xrightarrow{\text{CYP}} \text{Product (ROH)} + H_2O + \text{NADP}^+$$

Product from this reaction does not appear to be only a simple alcohol because rearrangement might be occurred. There are also documented that CYP may catalyze reduction reaction e.g. carbon tetrachloride, azo dyes and epoxides (Guengerich, F.P., 1991). However, biotransformation by CYP is not always a detoxification reaction. A variety of specific CYP isoforms, especially CYP is family 1, 2 and 3 are involved in the activation of certain chemical procarcinogens (Soucek, P., and Gut, I., 1992; Parkinson, A., 2001).

The majority of microsomal CYPs belong to families 1, 2, and 3 metabolize a great variety of structurally different xenobiotics such as drugs, alcohols, aromatic organic compounds, including many environmental pollutants and natural plant products. Some of these are chemical carcinogens or mutagens. These three families account for about 70% of total CYPs in human livers while CYP4 is a family of enzymes involved in fatty acid and prostaglandins metabolism (Rendic, S., and Di Carlo, F.J., 1997). CYP isoforms which play a role in the activation of xenobiotics to toxic metabolites include CYPs 1A1, 1A2, 2B1, 2B2, 2E1, 3A in rats as well as CYPs 1A1, 1A2, 2B6, 2E1, 3A4 in humans. An example of rat and human CYPs that activate some potential carcinogens/mutagens are demonstrated in Table 3.

The model system for study in CYPs was using rat, mouse and rabbit. Species differences have been noted in the expression of CYP gene. Table 4 presents sequential

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homology between rat and human CYP isoforms known. Sequential homology of cDNA and amino acid sequence between rat and human CYP isoforms is high in similarity, approximately 70%. There are generally conserved regions (for P-450 reductase, heam, signal peptide) which increase this similarity. On the other hand, change in a single amino acid may markedly or even completely alter CYP function, such as increase or decrease its activity, or even completely change its substrate specificity (Soucek, P., and Gut, I., 1992).

Important members of CYP families

CYP 1A Subfamily

CYP 1 family is composed of at least two subfamilies, CYP 1A and CYP 1B. *CYP 1A1* and *CYP 1A2* genes appear to be located in tandem on mouse chromosome 9, hamster chromosome 4 and human chromosome 15. Complete coding sequences have been determined for both CYP 1A1 and CYP 1A2 in human, dog, rabbit, mouse, rat and hamster. CYP 1A1 sequence has also been determined in monkey, guinea pig and fish (Ioannides, C., 1996).

Enzymes in CYP 1A subfamily are responsible for the metabolic activation of critical environmental chemicals such as polycyclic hydrocarbons, heterocyclic amine and aromatic amines. Substrate specifically metabolized by CYP 1A1 is benzo(a)pyrene [B(a)P], CYP 1A2 is acetanilide. These two forms of the CYP 1A subfamily are known to be induced by 3-methylcholanthrene (3-MC), β-naphthoflavone, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other polycyclic hydrocarbons. CYP 1A1 is expressed not only in the liver, but also in various extrahepatic tissues such as the lung and kidney, whereas the expression of CYP 1A2 is mainly confined to the liver. Then , exposure of man to the polycyclic hydrocarbons present in cigarette smoke differentially induces CYP 1A2 in the liver and CYP 1A1 in extrahepatic tissues, such as in lung (Ioannides, C., 1996; Gonzalez, F.J., and Gelboin, H.V., 1994; Oinomen, T., and Lindros, K.O., 1998).

CYP 1A1 and CYP 1A2 are found in both humans and rats (Soucek, P., and Gut, I., 1992). Function of CYP 1A is fairly well conserved across species, although there are subtle differences (Parkinson, A., 2001). For instance, isolated and purified human CYP 1A2 enzyme from the liver has been shown to display substrate specificity similar to the rat protein. The human CYP 1A2 isoform is functionally important, being in the metabolism of several clinically

important drugs such as acetaminophen, amitriptyline, caffeine, propranolol, theophylline, warfarin, etc. Some drugs are inducers (e.g. omeprazole, lansoprazole), or inhibitors (e.g. amiodarone, cimetidine, ticlopidine) of this CYP isoform (Parkinson, A., 2001; Rendic, S., and Di Carlo, F.J., 1997).

CYP 2B Subfamily

The CYP 2B subfamily is the subfamily of cytochrome P450 generally associated with phenobarbital-type induction in rodents. CYP 2B1 and CYP 2B2 are highly similar in nucleotide sequence and have similar substrate specificity. Rat CYP 2B1 is analogous to human CYP 2B6 (Soucek, P., and Gut, I., 1992). These enzymes are found in liver and extrahepatic tissues but are generally expressed at low levels in the absence of inducers such as phenobarbital. The CYP 2B6 cDNA was isolated from a human liver library and was found to metabolically activate 6-aminochrysene and 3-methoxy-4-aminoazobenzene. Its expression is quite variable in liver, ranging from about 2% of total P450 to nil (Gonzaleze, F.J., and Gelboin, H.V., 1994).

CYP 2E Subfamily

Cytochrome P450 2E1 (CYP 2E1) is constitutively expressed in liver and many other tissues. A cell-specific distribution implies and specific endogenous functions, although the level of CYP 2E1 in human liver is not impressively high compared to some other P450 isozymes. Within the cell, the highest concentrations of CYP 2E1 are found in the endoplasmic reticulum. Besides highest expressed in the liver, CYP 2E1 is also present in the kidney. Low levels of CYP 2E1 are expressed in many other tissues, including nasal mucosa, lung, testis ovaries, small intestine, colon, umbilical vein endothelial cells, lymphocytes, and brain (Ioannides, C., 1996). This enzyme is capable of metabolizing numerous low molecular weight chemicals, including benzene, carbon tetrachloride, acrylonitrite, N-nitrosodimethylamine, and others. The ubiquitous nature of CYP 2E1 substrates in the industrial workplace, environment, tobacco smoke, and diet has marked human health implication (Gonzaleze, F.J., and Gelboin, H.V., 1994). CYP 2E1 substrates such as ethanol, isopropanolol, acetone, toluene, and benzene may also induce CYP 2E1 itself. Isoniazid and imidazole compounds are also potent inducers. CYP 2E1 is also induced by the pathophysiologic state of diabetes (Rendic, S., and Di Carlo, F.J., 1997; Gonzaleze, F.J., and Gelboin, H.V., 1994). Human liver CYP 2E1 is similar to rat CYP 2E1 and

rabbit CYP 2E1 in structure, catalytic activity and regulatory characteristics (Wrighton, S.A., et al., 1986). Thus, CYP 2E1 are well conserved among mammalian species (Parkinson, A., 2001).

CYP 3A subfamily

The most abundantly expressed P450s in human liver belong to CYP 3A subfamily. Four P450s are known to exist in this subfamily: CYP 3A3, CYP 3A4, CYP 3A5 and the fetal CYP 3A5. Up to 60% of total P450 is due to CYP 3A. CYP 3A4 is the major form of P450 expressed in normal adult human liver microsomes and other tissues, including kidney, intestine and probably other tissues. CYP 3A4 representing approximately 30% of the spectroscopically detecable cytochrome P450 in the liver (Woolf, T.F., 1999). The CYP 3A are very important in metabolism of drugs and are involved in metabolic activation of a number of chemical carcinogens including aflatoxin B_1, benzo(*a*)pyrene 7,8-diol, 1-nitropyrene, 6-amino-chrysene, and the chemotherapeutic agent including morpholinodoxorubicin, cyclophosphamide, ifosfamide (Gonzaleze, F.J., and Gelboin, H.V., 1994). CYP 3A4 is the major P450 expressed in human and figures prominently in the metabolism of drugs. More than 150 drugs belonging to about 38 classes are listed as substrates of CYP 3A4. Examples of substrates are opioid analgesics, corticosteroids, immunosuppressants and antiarrhythmics. This enzyme also catalyzes the metabolism of endogenous steroids including androgens, anabolic hormones, cortisol, estradiol and progesterone (Table 2)

In human, CYP 3A enzymes are inducible by numerous drugs, including phenobarbital, phenytoin, rifampin and troglitazone. Inhibitors of human CYP 3A include azole type antimycotics (e.g. ketoconazole, clotrimazole), macrolide antibiotics (e.g. erythromycin), HIV protease inhibitors (e.g. ritonavir), ethynylestradiol, statin, and dihydropyridine calcium channel blockers (Parkinson, A., 2001).

Some dietary compounds can either stimulate or inhibit CYP 3A4 *in vivo* and *in vitro*. For instance, flavonoids (quercetin, kaempherol, tangeretin and naringenin) inhibit nifedipine and felodipine oxidation catalyzed by CYP 3A4 in human liver microsomes. Grapefruit juice (containing the flavonoids, quercetin, naringin, bergamottin and naringenin) was found to inhibit the oxidation of both nifedipine and felodipine as well as affect the disposition of cyclosporine, terfenadine, midazolam and 17 α-ethinylestradiol (Yang, C.S., *et al*., 1994).

CYP 3A1 and CYP 3A2 are found in rats. Expression of CYP 3A1 is very low in normal rat liver, whereas CYP 3A2 is the adult male-specitic CYP which is absent from the livers of adult females (Oinomen, T., and Lindros, K.O., 1998).

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Table 2 Human CYP enzymes, their specific substrates and their percent participation in drug metabolism (modified from Rendic, S., and Di Carlo, F.J., 1997)

CYP enzymes	Substrates	Participation in drug metabolism (%)
1A1	7-Ethoxyresorufin	2.5
	R-Warfarin	
1A2	Acetaminophen	8.2
	Caffeine	
	7-Methoxyresorufin	
	Phenacetin	
	R-Warfarin	
2B6	Cyclophosphamide	3.4
	7-Benzyloxyresorufin	
	S-Mephenytoin	
	7-Pentoxyresorufin	
	Testosterone	
2E1	Acetaminophen	4.1
	Aniline	
	Chlorzoxazone	
	Dapsone	
	Halothane	
	ρ-Nitrophenol	
2C8, 9	Diclofenac	15.8
	Hexobarbital	
	Phenytoin	
	Tolbutamide	
	S-Warfarin	

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Table 2 *(continued)* Human CYP enzymes, their specific substrates and their percent participation in drug metabolism (modified from Rendic, S., and Di Carlo, F.J., 1997)

CYP enzymes	Substrates	Participation in drug metabolism (%)
2C18, 19	Diazepam	8.3
	S-Mephenytoin	
	Omeprazole	
2D6	Codeine	18.8
	Bufuralol	
	Debrisoquine	
	Dextromethophan	
	Sparteine	
3A4, 5	Carbamazepine	34.1
	Cortisol	
	Dapsone	
	Diazepam	
	Erythromycin	
	Midazolam	
	Nifedipine	
	Omeprazole	
	Testosterone	

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Table 3 Role of rat and human CYPs in the activation of some potential carcinogens/ mutagens (Soucek, P., and Gut, I., 1992; Guengerich, F.P., 1992; Gonzalez, F.J., and Gelboin, H.V., 1994)

CYP	Potential mutagens/carcinogens	
	Rat	Human
1A1	Aflatoxin B_1	Benzo(a)pyrene
	Benzo(a)pyrene	7,12-Dimethylbenz(a)anthracene
	7,12-Dimethylbenz(a)anthracene	6-Nitrochrysene
	2-Naphthylamine	
	4,4'-(bis)Methylene chloroaniline	
1A2		2-Acetylfluorene
		2-Aminoanthracene
		Aflatoxin B_1
		4-Aminobiphenyl
		2-Naphthylamine
		6-Nitrochrysene
2B1	2-Acetylfluorene	
	Aflatoxin B_1	
	Benzo(a)pyrene	
	3-Methylcholanthrene	
	4,4'-(bis)Methylene chloroaniline	
2B2	4,4'-(bis)Methylene chloroaniline	
2B6		6-Aminochrysene
2B7		Aflatoxin B_1
2E1	N-N'-Nitrosodimethylamine	Acrylonitrile
	N-Nitroso-N-diethylamine	Benzene
		Carbon tetrachloride
		Chloroform
		N-Nitroso-N-diethylamine

Table 3 *(continued)* Role of rat and human CYPs in the activation of some potential carcinogens/mutagens (Soucek, P., and Gut, I., 1992; Guengerich, F.P., 1992; Gonzalez, F.J., and Gelboin, H.V., 1994)

CYP	Potential mutagens/carcinogens	
	Rat	Human
2E1		Styrene
		Trichloroethylene
		Vinyl carbamate
		Vinyl bromide
		Vinyl chloride
3A4		Aflatoxin B_1
		Aflatoxin G_1
		Benzo(a)pyrene
		6-Nitrochrysene
		Sterigmatocystin

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Table 4 Sequential homology between rat and human CYP forms (Soucek, P., and Gut, I., 1992)

P-450 form		Sequential homology[a] (%)
Rat	Human orthologue	
CYP 1A1	CYP 1A1	80(78)
CYP 1A2	CYP 1A2	75(70)
CYP 2A1	——	—[b]
CYP 2A2	——	—[b]
CYP 2A3	CYP 2A6	(85)
——	CYP 2A7	—[b]
CYP 2B1	CYP 2B6	78(74)
	CYP 2B7	(76)
CYP 2B2	——	—[b]
CYP 2B3	——	—[b]
CYP 2C6	CYP 2C10	(75)
CYP 2C11	CYP 2C9	80(77)
CYP 2C12	——	—[b]
CYP 2C13	CYP 2C8	74(68)
CYP 2D1	CYP 2D6	(71)
CYP 2E1	CYP 2E1	75(78)
CYP 3A1	CYP 3A3	(78)
——	CYP 3A4	(73)
CYP 3A2	CYP 3A5	(71)
	CYP 3A7	(65)
CYP 3A9	——	—[b]
CYP 4A1	CYP 4A9	—[b]
CYP 4A3	——	—[b]
CYP 11A1	CYP 11A1	79(76)

[a] Similarity of cDNA and amino acid (in parentheses) sequence stated.

[b] No data available regarding existence of orthologous form.

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Mechanism of induction of CYPs

Induction is defined as an increase in amount and catalytic activity of CYP. Although the precise molecular mechanisms of cytochrome P450 induction are not fully understood at present, much effort has been expended in trying to rationalize the inductive response of the drug-metabolising enzymes in hepatic tissue. Figure 3 shows the functional components of the hepatic mixed function oxidase system responsible for cytochrome P450-dependent drug metabolism. Accordingly, induction of drug metabolism may arise as a consequence of increased synthetis, decreased degradation, activation of pre-existing components or a combination of these three processes. More specifically, Table 5 summarises some of the biochemical effects noted on response to enzyme inducers. From this table, it is clear that enzyme inducers have a variety of effects on the functional components of the mixed function oxidase system, particularly on the terminal haemoprotein, cytochrome P450 (Gibson, G.G., and Skett, P., 1994).

Table 5 Differences in induction mechanisms for cytochrome P450s

P450 isoenzymes	Representative inducer	Main induction mechanism
1A1	Dioxin	Transcriptional activation by ligand activated Ah receptor
1A2	3-Methylcholanthrene	mRNA stabilization
2B1/2B2	Phenobarbital	Transcriptional gene activation
2E1	Ethanol, acetone, isoniazid	Protein stabilization (in part)
3A1	Dexamethasone	Transcriptional gene activation
4A6	Clofibrate	Transcriptional activation, mediated by peroxisome proliferators activated receptor

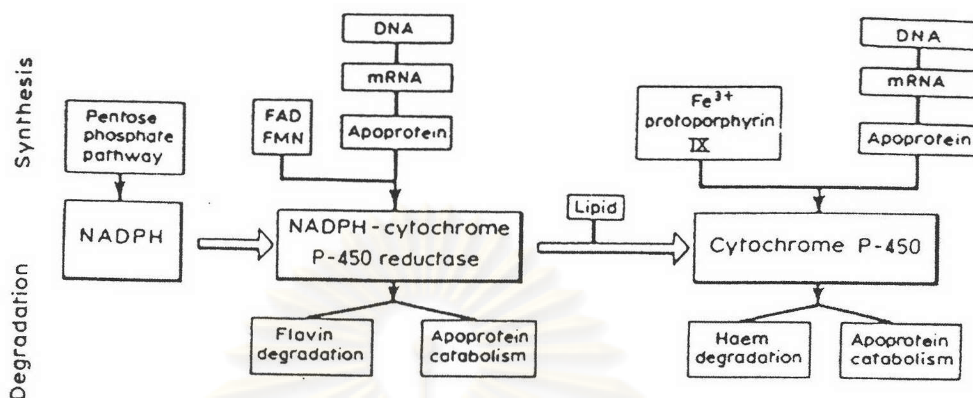

Figure 3 Synthesis and degradation of the functional components of the hepatic mixed function oxidase system.

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Mechanism of inhibition of CYPs

(Gibson, G.G., and Skett, P., 1994; Lin, J.H., and Lu, A.Y.H., 1998; Woolf, T.F., 1999)

Enzyme inhibition results in a decrease in the clearance, thereby an increase in the steady-state serum concentration of the affected drug. A major concern of clinical pharmacologists is the area of drug-drug interaction in which two or more drugs are co-administered resulting in either therapeutic incompatibility or toxic reactions. Just as one drug can induce the metabolism of a second drug as discussed in the section of induction of CYPs, the inhibition of drug metabolism by other drugs or xenobiotics is a well-recognised phenomenon. Inhibition of CYP enzymes is the most common cause of inhibition of drug metabolism, that is clinical importance for both therapeutic and toxicological reasons. The mechanisms of CYP inhibition can be divided grossly into 3 categories: reversible inhibition, quasi-irreversible inhibition and irreversible inhibition.

1. Reversible inhibition

Reversible inhibition is the most common mechanism responsible for the documented drug interactions. Reversible inhibition can be further classified into competitive, uncompetitive, mixed-type and non-competitive inhibition. Competitive inhibition is when the binding of an inhibitor to an enzyme prevents a further binding of a substrate to the active sites of the enzyme. In uncompetitive inhibition, an inhibitor does not bind to the free enzyme, but binds to the enzyme-substrate complex, resulting in a nonproductive enzyme-substrate-inhibitor complex. Mixed-type inhibition is when an inhibitor binds either to the free enzyme or to the enzyme-substrate complex. Non-competitive inhibition, an inhibitor binds to a nonactive binding site of the enzyme, and the binding has no effect on the binding of substrate, but the enzyme-substrate-inhibitor complex is nonproductive.

Many of the potent reversible CYP inhibitors are nitrogen-containing drugs, including imidazoles (e.g. ketoconazole, cimetidine), pyridines (e.g. metyrapone, indinavir) and quinolines (e.g. quinidine, primaquine, chloroquine, amodiaquine, mefloquine). The potency of an inhibitor is determined both by its lipophilicity and by the strength of the bond between its nitrogen lone pair electron and the prosthetic haem iron.

2. Quasi-irreversible inhibition *via* metabolic intermediate complexation

A large number of drugs, including methylenedioxybenzenes, alkylamines, macrolide antibiotics and hydrazines, undergo metabolic activation by CYP enzymes to form inhibitory metabolites. These metabolites can form stable complexes with the prosthetic haem of CYP, called metabolic intermediate (MI) complex, so that the CYP is sequestered in a functionally inactive state. MI complexation can be reversed, and the catalytic function of ferric CYP can be stored by *in vitro* incubation with highly lipophilic compounds that displace the metabolic intermediate from the active site. Dissociation or displacement of the MI complex results in the reactivation of CYP functional activity. However, in *in vivo* situations, the MI complex is so stable that the CYP involved in the complex is unavailable for drug metabolism, and synthesis of new enzymes is the only means by which activity can be restored. The nature of the MI complexation is, therefore, considered to be quasi-irreversible.

3. Irreversible inhibition

Drugs containing certain functional groups can be oxidized by CYP to reactive intermediates that cause irreversible inactivation of the enzyme prior to its release from the active site. Because metabolic activation is required for enzyme inactivation, these drugs are classified as mechanism-based inactivators or suicide substrates. The mechanism-based inactivation of CYP may result from irreversible alteration of haem or protein, or a combination of both. The most important phenomena of mechanism-based inhibition of CYP are time, concentration, and NADPH-dependent loss of the enzyme activity. *In vivo*, the inhibitory effect of a mechanistic inactivator is thought to be more prominent after repeated dosing and last longer than that of a reversible inhibitor.

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