CHAPTER II

LITERATURE REVIEW

Flower resources are abundant in Thailand, of which many kinds are edible and can be used as herbs. People in this region have long history of consuming flowers in dietary food (Institute of nutrition, 1999). Some flowers have been used as food and for medicinal purpose for centuries. There were many studies of flowers *in vitro* and *in vivo*. Chewonarin *et al.* (1999) reported that the 80% ethanol extract of roselle flower (*Hibiscus sabdariffa* Linn.) reduced about 60-90% of the mutagenicity induced by 2-amino-1-methyl-6-phenylimidazo[4,5*b*]pyridine (PhIP) and other heterocyclic amines 2-amino-3-methylimidazo[4,5*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5*f*] quinoxaline (MeIQx), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (Glu-P-1), 2-aminodipyrido[1,2-*a*:3,2'-d] (Glu-P-2), at a concentration of 12.5 mg/plate in the *Salmonella* mutation assay. In addition, Busayaskul (2006) assayed the raw and conventional processed (boiled, battered and fried) samples of eight edible flowers namely hua-plee (*Musa sapientum* Linn.), dok-khachon (*Telosma minor* Craib.), dok-khem (*Ixora coccinea* Linn.), dok-khae (*Sesbania grandiflora* Desv.), dok-bualuang (*Nelumbo nucifera* Gaertn.), dok-fueangfa (*Bougainvillea glabra* Choisy.), dok-sano (*Sesbania javanica* Miq.), and dok-anchan (*Clitoria ternatea* Linn.) had antimutagenicity using somatic mutation and recombination test (SMART).

In addition, active substances from flowers were polyphenol group e.g. flavonoids, anthocyanins. Anthocyanins belong to the flavonoid compound and, as coloured flavonoids, are prominent in flower petals and fruit peels (Brouillard and Dangles, 1994).

2.1 Major Natural Components in Flower

2.1.1 Flavonoids

Flavonoids, or bioflavonoids, are a ubiquitous group of polyphenolic substances which are present in most plants, concentrating in seeds, fruit skin or peel, bark, and flowers. A great number of plant medicines contain flavonoids, which have been reported by many authors as having antibacterial (Havsteen, 1983) anti-inflammatory (Kim *et al.*, 1998), antimutagenic (Edenharder *et al.*, 2001), antiviral (Thomas, Nash and Dormandly, 1988), antineoplastic (Hirano, Gotoh and Oak, 1994), anti-thrombotic (Lou *et al.*, 1989) and vasodilatory actions (Marchand, 2002). Flavonoids may be divided into six different major classes (flavonols, flavanones, flavones, isoflavones, flavonols and anthocyanidins) based on differences in molecular backbone structure. The major classes of flavonoids consist of two fused six-membered rings (an aromatic A-ring and a heterocyclic C ring) connected through a carbon-carbon bridge to an aromatic B-ring. The structural components common to these molecules include two benzene rings on either side of a 3-carbon ring (see Figure 1). Multiple combinations of hydroxyl groups, sugars, oxygens, and methyl groups attached to these structures create the various classes of flavonoids: flavanols, flavanones, flavones, flavan-3-ols (catechins), anthocyanins, and isoflavones. Flavonoids have been shown in a number of studies to be potent antioxidants, capable of scavenging hydroxyl radicals, superoxide anions, and lipid peroxy radicals (Hoensch and Kirch, 2005). The impact of flavonoids on various mechanisms and functions relevant for the molecular biology are listed in Table 1.

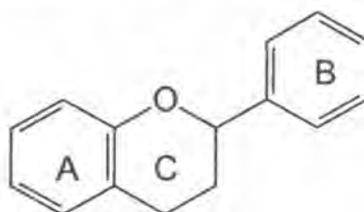

Figure 1. Basic monomeric structure of flavonoids

Table 1 Biomolecular activities of flavonoids

- Antioxidative effect: inactivation of oxygen radicals
- Binding of electrophils
- Induction of protective enzymes: phase 2 enzymes with conjugating activities (GT, GST)
- Apoptosis rate increase
- Cell proliferation inhibition
- Lipidperoxidation inhibition
- Angiogenesis inhibition
- H-Donation (e.g. GSH-peroxidase)
- DNA oxidation inhibition

GT, glucuronosyl transferases; GST, glutathione S-transferases; GSH, glutathione. (Hoensch and Kirch, 2005).

Human ingest about 1 g of flavonoids daily in their diet, and they are increasingly associated with cytoprotective antitumor properties. The mechanism responsible for these effect have not yet been elucidated but may involve interaction with xenobiotic metabolizing enzymes to alter the metabolic activation of potential carcinogens (Rodgers and Grant, 1998). Some flavonoids cause apoptosis in human tumor cells and act as antiproliferative agents in cancer cells (Middleton, Kandaswami, and Theoharides, 2000)

Flavonols from brussels sprouts and flavones can induce protective enzymes such as conjugating enzymes, e.g., UDP-glucuronosyl transferases, glutathione S-transferases in gut and liver (Nijhoff, Groen and Peters, 1993; Vanderlogt *et al.*, 2003). These enzymes inactivate electrophiles, free radicals, and reactive oxygen species thereby preventing them from becoming mutagens (Steele, Kelloff, and Balentine, 2000). The potential mechanisms of inhibition of carcinogenesis by flavonoids are demonstrated in Figure 2 (Hoensch and Kirch, 2005). Figure 2 illustrates the inhibitory effects of tea flavonoids on the main biological events that can lead to mutagens and shows how the carcinogenic neoplastic processes (initiation, promotion, and progression) are influenced. These flavonoids can have an upstream effect in this cascade of carcinogen by trapping and inactivating reactive oxygen

species, electrophils, and free radical compounds. Other flavonoids are also effective further downstream by inhibiting enzymes that mediate inflammatory reaction (COX2, LOX2). Experimental evidence suggests that tea polyphenols also suppress enzymes of cell division and proliferation such as ornithine decarboxylase, tyrosine kinase, telomerase, and topoisomerase (Hoensch and Kirch, 2005)

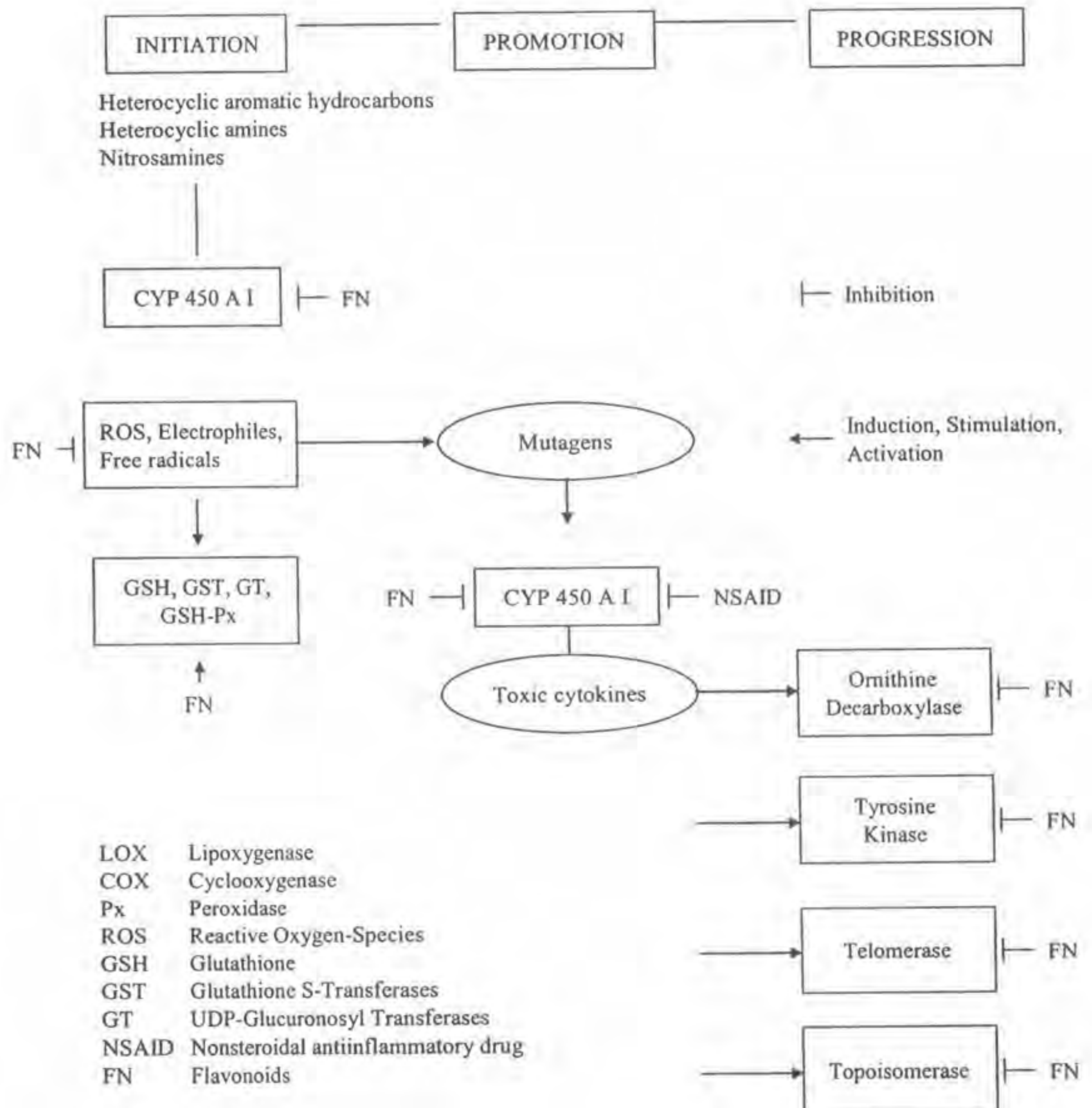

Figure 2. Hypothesis of inhibition of carcinogenesis by flavonoids (FN) (Hoensch and Kirch, 2005).

2.1.2 Anthocyanins

Anthocyanins are flavonoids (flavan-like) commonly found in nature. They are the most important pigments of the vascular plants; they are harmless and of easy incorporation in aqueous media, which makes them interesting for their use as natural

water-soluble colorants (Pazmiño-Durn *et al.*, 2001). These pigments are responsible of the shiny orange, pink, red, violet and blue colours in the flowers and fruits of some plants.

The anthocyanidins are the basic structure of the anthocyanins (figure 3). The anthocyanidins (or aglycons) consist of an aromatic ring [A] bonded to an heterocyclic ring [C] that contains oxygen, which is also bonded by a carbon-carbon bond to a third aromatic ring [B] (Konczak and Zhang, 2004). When the anthocyanidins are found in their glycoside form (bonded to a sugar moiety) they are known as anthocyanins.

The compounds which are easier to be oxidized are often the best antioxidants (molecules which can donate a free electron or hydrogen atoms to reactive free radicals). Several studies have suggested that the anthocyanin content and their corresponding antioxidant activity, contribute to the fruits and the vegetables protective effect against degenerative and chronic diseases (Heinonen, Meyer and Frankel, 1998; Record, Dreosti and Mcinerney, 2001). In addition, Gasiorowski *et al.* (1997) established that anthocyanins isolated from fruits of *Aronia melacarpa* markedly inhibited the mutagenic activity of benzo[a]pyrene and 2-amino fluorine in the Ames test.

Shih, Yeh and Yen (2005) initially demonstrated the ability of anthocyanins to induce phase II antioxidant and detoxifying enzymes in cultured cells. Treatment of rat liver clone 9 cells with 50 μM anthocyanins (Shih, Yeh and Yen, 2007) and non-cancerous breast cells with 10-20 μg/ml anthocyanins (Singletary, Jung and Giusti, 2007) enhanced their antioxidant capacity by activating glutathione-related enzymes (glutathione reductase, glutathione peroxidase, and glutathione S-transferase) as well as the activity of NAD(P)H: quinone reductase.

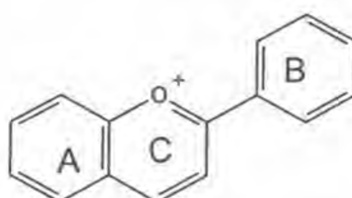

Figure 3. Structural identification of anthocyanidins (aglycons)

2.1.3 Carotenoids

Carotenoids are the pigments responsible for the color of many plants, fruits, and flowers. They are present in a wide variety of food and feeds such as yellow and green vegetables, tomatoes, apricots, oranges, peppers, egg yolk, chicken, butter, shrimp, lobsters, trout, and yellow corn (Tee, 1992). In addition, they are found in marigold flower (*Tagetes erecta*). Carotenoids are important in human nutrition as a source of vitamin A and as a prevention agent for cancer and heart disease. They have excellent antioxidant properties; α or β carotene, xanthophylls (mainly lutein and its isomers), and retinoids have been reported to inhibit some types of cancers (Moon, McCormick and Metha, 1983; Krinsky, 1994).

Yellow flowers most commonly derived their colour from carotenoid pigmentation. However, they also confer various transition colors in conjunction with other pigments. For instance, various colour ranges and tones of orange are generated by the co-existence of yellow pigments with different amounts of anthocyanins. Some yellow acyanic (lacking any red pigments, e.g., anthocyanins and betacyanins) flower colours arise from yellow flavonoids (such as aurones, chalcones), carotenoids, and betaxanthins. Moreover, there are few reports of flowers that yellow colour derived from colourless flavonoids such as the flavonols, kaempferol and quercetin, e.g. *Lathyrus chrysanthus, Camellia chrysantha* and *Eustoma grandiflorum* (Harborne, 1965; Markham; Gould and Ryana., 2001; Yoshida *et al*., 2004)

The studies presented by González de Mejia, Ramos-Gmez and Loarca-Piña (1997) indicated that the carotenoids present in Aztec Marigold (*Tagetes erecta*) had a strong inhibitory effect on mutagenicity of aflatoxin B1 using the *Salmonella typhimurium* strain YG 1024 in the plate-incorporation test.

2.1.4 Chlorophyll

All green plants contain chlorophyll, the light-collecting molecule. Chlorophyllin is a water-soluble derivative of chlorophyll, which has been intensively studied as an inhibitor to various mutagens (Sarkar, Sharma and Talukder, 1994). Chlorophyll and its derivatives are very effective in binding polycyclic aromatic hydrocarbons (carcinogen largely from incomplete combustion of fuels), heterocyclic amines (generated when grilling foods), aflatoxin (a toxin from molds in foods which causes liver cancer), and other hydrophobic molecules (Donaldson, 2004). The chemoprotective effect of chlorophyll and its derivatives has been tested in laboratory

cell cultures and animals (Chernomorsky, Segelman and Poretz, 1999.; Sarkar, *et al.*, 1994)

Mechanisms of the antimutagenic activity of chlorophyllin towards benzo[a]pyrene (B[a]P) were studied in vitro. In the *Salmonella* assay, chlorophyllin inhibited the mutagenic activity of B[a]P in the presence of S 9 activation system and was particulary effective against the direct-acting ultimate carcinogen, benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide. Dose-related inhibition of several cytochrome P450-dependent enzyme activities was observed upon addition of chlorophyllin to *in vitro* incubations. Spectral changes for the interaction between chlorophyllin and cytochrome P450 indicated that chlorophyllin does not bind to the active site of the enzyme, but exerts its inhibitory effect indirectly. This was achieved by inhibiting NADPH-cytochrome P450 reductase, and did not involve lowering of the effective substance concentration by complex formation with the procarcinogens (Tachino *et al.*, 1994)

2.1.5 Triterpenoids

Triterpenoids, synthesized in many plants by the cyclization of squalene, are widely used in Asian medicine (Liby, Yore and Sporn, 2007). More than 20,000 triterpenoids are known to occur in nature, two of these, oleanolic acid and ursolic acid, are weakly anti-inflammatory and anti-tumorigenic *in vivo* (Ovesna *et al.*, 2004; Huang, 1994). Oleanolic acid and ursolic acid are found in plants worldwide. Saraswat *et al.* (1996) suggested that ursolic acid inhibited metabolic activation of procarcinogen and the toxic metabolite formation catalyzed by CYPs, which is one of the mechanisms of carcinogenesis inhibition and hepatoprotective effects.

In addition, maslinic acid a natural triterpene from pomegranate flower exhibited antioxidant activity by decreasing conjugated diene (CD) production of low-density lipoprotein (LDL) susceptibility to oxidation in the rat brain tissue in vitro. Moreover, maslinic acid inhibited cell proliferation significantly in a dose-dependent manner and caused apoptotic death in HT-29 colon cancer cells (Reyes-Zurita *et al.*, 2009).

2.2 Selected Flower

2.2.1 *Hibiscus rosa-sinensis* Linn. (Red hibiscus, ชบา): MALVACEAE

Hibiscus rosa-sinensis is an ornamental plant throughout the tropics and subtropics (Figure 4). The flowers are large, red, firm, but lack any scent. Numerous varieties, cultivars and hybrids are available, with flower colors ranging from white through yellow and orange to scarlet and shades of pink, with both single and double sets of petals.

Pharmacological Activity of Red Hibiscus Flower

Hibiscus rosa-sinensis is used in traditional medicines in a number of countries including India, China, Japan, parts of Africa and America for the treatment of cough, fever, dysentery, veneral disease, as an abortifacient and is also applied topically to cancerous swellings (Ross, 1999). In medicine, the red flowered variety is preferred. Flowers have been found to be effective in the treatment of arterial hypertension (Dwivedi, Pandey, and Tripathi., 1977) and to have significant antifertility effect (Singh, Singh, and Udupa, 1982; Sethi, Nath, and Singh., 1986). In mouse, oral administration of benzene extract of *Hibiscus rosa-sinensis* flowers at a dose level of 1 g/kg body weight/day 5-8 of gestation led to termination of pregnancy in about 92% of the animals (Pakrashi *et al.*, 1986). In addition, Nivsarkar *et al.* (2005) studied antiimplantation activity of water extract of leaves of *Hibiscus rosa-sinensis*. They found that no implantation sites in pregnant female mice were received the extract (100mg/kg body weight) from days 1 to 6 of pregnancy. Other studies of *Hibiscus rosa-sinensis*, it was found that petroleum ether extract of leaves and flowers of *Hibiscus rosa-sinensis* showed its potential on hair growth by *in vivo* and *in vitro* methods. *In vivo*, 1% extract of leaves and flower in liquid paraffin was applied topically over the shaved skin of albino rats and monitored and assessed for 30 days. In vitro, the hair follicles from albino rat neonates were isolated and cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 0.01 mg/ml petroleum ether extract of leaves and flowers. From the study it was concluded that the leaf extract, when compared to flower extract, exhibited more potency on hair growth (Adhirajan *et al.*, 2003). Moreover, Sachdewa and Khemani (2003) revealed that oral administration of an ethanol flower extract of *Hibiscus rosa-sinensis* lowered the total cholesterol and serum triglyceride in streptozocin induced diabetic rats by 22

and 30%, respectively. It possessed various pharmacological activities such as radical scavenging, antipyretic, and anti-inflammatory activities (Singh *et al.*, 1978; Masaki *et al.*, 1995). Sharma and Sultana (2004) proposed that *Hibiscus rosa-sinensis* extract exerted a protective effect against the tumour promotion stage of cancer development. The study was conducted to investigate the ameliorative potential of *Hibiscus rosa-sinensis* extract in mice skin.

The constituents presented in extract of *Hibiscus rosa-sinensis* are quercetin, carotene, niacin, riboflavin, malvalic acid, gentisic acid, margaric acid and lauric acid (Ross, 1999). Sharma, Khan and Sultana (2004) studied the role of gentisic acid, which is a phenolic acid, in the chemopreventive activity of *Hibiscus rosa-sinensis* extract on 7,12-dimethyl benz(a)anthracene (DMBA)/croton oil-mediated carcinogenesis in mouse skin via 12-*O*-tetradecanoyl phorbol-13-acetate (TPA)-induced tumour promotion response and oxidative stress. A single topical application of TPA caused significant depletion in reduced glutathione (GSH) content, activities of its metabolizing and antioxidant enzymes, while malondialdehyde (MDA) formation, H_2O_2 content, ornithine decarboxylase (ODC) activity and DNA synthesis were significantly increased. Pretreatment of *Hibiscus rosa-sinensis* extract (3.5 mg and 7 mg/kg body weight) and gentisic acid (2.0 and 4.0μg/0.2 ml acetone per animal) restored the level of GSH and its metabolizing and antioxidant enzymes ($p<0.05$). There was also a statistically significant reduction in MDA formation and H_2O_2 content ($p<0.05$) at both doses. Moreover, Nayak *et al.* (2007) reported that *Hibiscus rosa-sinensis* aided wound healing in the rat model. Animals treated with the extract of *Hibiscus rosa-sinensis* exhibited an 86% reduction in the wound area compared with controls, who exhibited a 75% reduction.

Figure 4. *Hibiscus rosa-sinensis* Linn. (Red hibiscus, ชบา)

2.2.2 *Antigonon leptopus* Hook. & Arn (Mexican creeper, พวงชมพู): POLYGONACEAE

The tubers and flowers of *Antigonon leptopus*, (Figure 5) belonging to the family Polygonaceae, are consumed as food in several parts of the world. *Antigonon leptopus* is native of Mexico and commonly found in tropical Asia, Africa, Caribbean and the Americas (Raju *et al.*, 2001). It is a fast growing, evergreen vine, climbing with tendrils that will reach 40 feet (13 meters). It bears masses of deep pink, heart-shaped flowers from early summer to fall (autumn), and is ideal for trellises, pergolas and arbours where a light cover is desirable. In Thailand, the fried flour-coated leaves and flowers of *Antigonon leptopus* are served with noodles. The flowers are also used in omelets (Charmaine, 1998).

Pharmacological Activity of Mexican Creeper Flower

A hot tea prepared from the aerial portion of this plant extracts, was used as a treatment for cough and throat constriction in Jamaica and considered as one of the important medicinal plants in their folk-medicine (Mitchell and Ahmad, 2006). Methanol extract of *Antigonon leptopus* demonstrated activity of 89 % in the inhibition of thrombin (Chistokhodova *et al.*, 2002). In addition, Lans (2006) revealed that vine part of *Antigonon leptopus* exhibited anti-diabetic activities. Other studies, Vanisree *et al.*, 2008 found that the methanol extract of the aerial parts of *Antigonon leptopus* inhibited lipid peroxidation (LPO) by 89% and cyclooxygenase enzymes, COX-1 and COX-2 by 50.4% and 72.5%, respectively, at 250 μg/ml. Purification of the methanolic extract yielded *n*-hentriacontane, ferulic acid, 4-hydroxycinnamic acid, quercetin-3-rhamnoside, and kaempherol-3-glucoside along with β-sitosterol, β-sitosterol-glucoside and d-mannitol. Compounds 4-hydroxycinnamic acid, quercetin-3-rhamnoside, and kaempherol-3-glucoside inhibited lipid peroxidation by 19.5%, 41.0% and 60.5% respectively, at 5 μg/ml. Similarly, compounds 4-hydroxycinnamic acid, quercetin-3-rhamnoside, and kaempherol-3-glucoside inhibited COX-1 enzyme by 64.7%, 16.9% and 38.5% and COX-2 enzyme by 87.4%, 88.8% and 90.2%, respectively, at 25 μg/ml. In addition, the ethanol extract of the air-dried flowers of *Antigonon leptopus*, quercetin, rhamnetin, quercetin-3-O-β-D-glucopyranoside and a new anthraquinone glycoside, 1,5-dihydroxy-3-methylanthraquinone-8-*O*-(4-*O*-α-L-arabinofuranosyl)-β-D-glucopyranoside were isolated and characterized (Tiwari and Minocha, 1980). Moreover, Purachat 2005 found potassium 94.51 mg/kg in

Antigonon leptopus by flame-atomic absorption spectrometer and X-ray fluorescence spectrometer.

Figure 5. *Antigonon leptopus* Hook. & Arn (Mexican creeper, พวงชมพู)

2.2.3 *Ixora coccinea* Linn. (Ixora, เข็ม): RUBIACEAE

Ixora coccinea is one of the world's most popular tropical flowering shrubs (Figure 6). It is used for hedges and borders, for accent plants, in planters and as indoor potted plant. For food, the red flower is commonly in the dish because it makes nice looking. It may be added into sour curry or Kang Som, battered, or eaten raw (ทยา, 2545; สุพัตรา, 2548).

Pharmacological Activity of Ixora Flower

The flowers of *Ixora coccinea* have been used in traditional Indian systems of medicine for dysentery, healing of ulcers and, more recently, for an anti-tumour activity (Latha and Panikkar, 1998). Latha and Panikkar, 1998 studied the cytotoxic properties of the *Ixora coccinea* flowers to murine tumour cells and antitumor activity against transplantable tumours in mice. They showed that the active fraction of flower contained a triterpenoid that exhibits antitumour property. From a methanol extract of the flowers of *Ixora coccinea*, 13 chemicals were identified, including ursolic acid (triterpenoid), which has known antitumour and antiviral activity (Francis, 2000). It also has been suggested to have a hepatoprotective effect against chemical-induced hepatic injury (Saraswat *et al.*, 1996). In addition, ursolic acid was tested for its ability to modulate the activities of several cytochrome P450 enzymes. Kim *et al.* (2004) demonstrated that ursolic acid had an inhibitory effect on CYP2C19 in human liver microsomes. Subramanian and Nair (1971) found that ixora flower contained flavonoids (cyanidin-3-rutinoside (traces) and leucocyanidin glycoside).

In 2001, Latha and Panikkar studied the protective effect of the active fraction of *Ixora coccinea* flowers against cisplatin induced toxicity in mice. It helped to prolong the life span of the animals treated with cisplatin. Moreover, the alcoholic extract of the flowers of *Ixora coccinea* was studied for its effect on the various parameters concerned with the process of healing. It increased in granuloma tissue weight, tensile strength, hydroxyproline and glycosaminoglycan content on wound healing, using a dead space wound model in rats (Nayak, Udupa and Udupa, 1999).

A decoction of flowers was used as a lotion to relieve eye troubles and for sores, chronic ulcers, scabies and some types of dermatitis.

Figure 6. *Ixora coccinea* Linn. (Ixora, เข็ม)

2.2.4 *Plumeria obtusa* Linn. (White frangipani, ลั่นทมขาว): APOCYNACEAE

Plumeria obtusa belongs to the Apocynaceae (Figure 7). It is a native of the Bahama islands, Cuba, Jamaica, Hispaniola and Pureto Rieo. Plumeria flowers are excellent lei flowers and are especially common and traditional for home-made leis. All parts of the plant exude a milky sap when damaged. The sap may irritate eyes and skin.

Pharmacological Activity of White Frangipani Flower

Various species of this genus were reported for their medicinal uses in the indigenous system of medicine (Siddiqui and Begum, 1999). An ethanol crude extract of the *Plumeria obtusa* flower was found to contain a large amount of β-glucoside. An iridoid β-glucoside with two glucosyl groups attached, namely plumieride coumarate glucoside, was subsequently isolated from *Plumeria obtusa* (Boonclarm *et al.*, 2006). Plumieride coumarate glucoside can be detected not only in

flowers but also in other tissues of *Plumeria obtusa*, such as leaf and stem, but in lower levels.

Figure 7. *Plumeria obtusa* Linn. (White frangipani, ลั่นทมขาว)

2.2.5 *Syzygium malaccense* (Linn.) Merr. & Perry (Malay apple, ชมพู่ม่าเหมี่ยว): MYRTACEAE

The *Syzygium malaccense* is much admired for the beauty of the tree, its flowers and its red, glistening fruit (Figure 8) (Sankat, Basanta and Maharaj, 2000). Flowering is usually seasonal, fom 1-2 months in duration, but it varies widely from place to place and the even from year to year. In some areas, *Syzygium malaccense* flowers two or three times per year (Whistler and Elevitch, 2006). Clusters of cream or reddish purple flowers occur on branches or trunk, in summer.

Pharmacological Activity of Malay Apple Flower

Syzygium malaccense is used in the traditional medicine of Western Samoa for treating disorder of inflammatory nature, including inflammation with fever, sore throat, boils (skin abscess), wounds, skin ulcers, and mouth and throat infections (Noreen *et al.*, 1998). A bark decoction was drunk to ease stomachache and diarrhea (Ahmad and Ismail, 2003). Methanol or acetone extract of leaves of *Syzygium malaccense* inhibited cyclooxygenase-1 catalysed prostaglandin biosynthesis in vitro. It showed moderate inhibition (46%) (Dunstan *et al.*, 1997). In addition, (+)-Catechin, (+)-gallocatechin and 4′-O-methyl-*ent*-gallocatechin were obtained from the leaves of *Syzygium malaccense* (Dixon Xie and Sharma, 2005). Other studies, the water extract from the bark of *Syzygium malaccense* had antiviral selectivity indices (50 % cytotoxic concentration/ 50 % effective antiviral concentration) of 109. The

extract showed complete cell protection against HIV-induced cytopathic effect compared with control samples (Locher *et al.*, 1996). Ethanol extract of leaves or natural product powder of *Syzygium malaccense* inhibited aldose reductase 82%. Aldose reductase, the key enzyme of the polyol pathway, was known to play important roles in diabetic complications (Guzman and Guerrero, 2005). There was no study of Malay apple flower.

Figure 8. *Syzygium malaccense* (Linn.) Merr. & Perry (Malay apple, ชมพู่มะเหมี่ยว)

2.2.6 *Curcuma sessilis* Gage. (Kra chiew, กระเจียว): ZINGIBERACEAE

The curcuma genus belongs to the Zingiberaceae family and is native to tropical Asia. It is used for food (Figure 9).

Figure 9. *Curcuma sessilis* Gage. (Kra chiew, กระเจียว)

2.2.7 *Nelumbo nucifera* Gaertn.(Sacred lotus, บัวหลวง): NYMPHAEACEAE

Nelumbo nucifera, commonly known as lotus, is a perennial aquatic herb widely found in tropical Asia to Australia (Figure 10). Lotus is used not only as an ornamental plant, but also consumed as food throughout Asia. The young leaves and flower stalks, seeds and rhizomes are all edible. Lotus flowers could be made into liquor and tea in folk diet (Lee *et al.,* 2005; Toyoda *et al.*, 1997).

Pharmacological Activity of Sacred lotus Flower

The plant has been known for a long time in Ayurvedic literature as an antipyretic, diuretic, as an astringent remedy in diarrhea and as an aphrodisiac. The petals have also been found useful in the therapies of hematemesis, eczema, weak spleen and stomach trouble (Li, 2003). Phoolphithayadhorn (2001) investigated the possible modulating effects of various products derived from lotus (*Nelumbo nucifera*) using SMART, the result showed that rhizome lotus drinks were antimutagenic and no products were toxic or mutagenic. Moreover, ethanol (100%) and water extracts of dried flower, administered intragastrically to rabbits at a dose of 1.0 g/kg were active in hypoglycemic activity. It was revealed that extracts of the dried flower have a marked hypoglycemic action, significantly improve glucose tolerance and suppress the hyperglycemic effect of adrenaline in normal rabbits (Huralikuppi, Christopher and Stephen, 1991(a), 1991(b)).

Flower contained flavonoids (kaempferol-3-glycoside) (ถนอมศรี, 2538). Yang *et al.* (2008) studied anthocyanins and flavonoids in petal of lotus cultivars by high-performance liquid chromatography with photodiode array detection/ mass spectrometry. They found five anthocyanins (delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, petunidin 3-O-glucoside, peonidin 3-O-glucoside and malvidin 3-O-glucoside) and ten flavonoids (quercetin 3-O-rutinoside, quercetin 3-O-galactoside, quercetin 3-O-glucuronide, kaempferol 3-O-robinobioside, kaempferol 3-O-galactoside, isorhamnetin 3-O-rutinoside, kaempferol 3-O-glucoside, kaempferol 3-O-glucuronide, syringetin 3-O-hexose and kaempferol 3-O-pentose) in lotus petals. Moreover, the hot water and methanol extracts of dried lotus flower demonstrated inhibitory effect against the activity of reverse transcriptase, the enzyme in the one stage of the synthesis of viral DNA, using Moloney Murine Leukemia virus enzymes as the test enzyme (Suthienkul *et al.*, 1993). In India, hot water extract of the dried flower was taken orally for cholera (Ross, 2005). In addition, water extract of lotus

stamen inhibited growth of *Staphylococcus aureus* (Avirutinant, and Pongpan 1983). Although the 95% ethanol extract of dried lotus stamen inhibited growth of *β-streptococcus* group A but it did not inhibit the growth of *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (อารีรัตน์, สุรัตนา และ วิเชียร, 2532).

Figure 10. *Nelumbo nucifera* Gaertn. (Sacred lotus, บัวหลวง)

2.2.8 *Millingtonia hortensis* Linn. (Indian cork tree, ปีป): BIGNONIACEAE

Millingtonia hortensis is an important medicinal plant in Southeast Asia, ranging from India, Burma, Thailand, Vietnam, Southern China and Indonesia (Figure 11). In Thailand, the flower is called 'peep'

Pharmacological Activity of Indian Cork Tree Flower

The plant has been claimed in Thai folk medicines to be useful for the treatment of tuberculosis, asthma and sinusitis (Takashi, Kazuhiro and Ryoji, 1995). From the dried flower of *Millingtonia hortensis*, cyclohexylethanoids and related glucosides were isolated (Hase *et al.*, 1995) There were reported of mutagenicity and antimutagenicity of hispidulin (6-methoxy-5,7,4'-trihydroxyflavone) and hortensin (3,4'-dihydroxy-6,7-dimethoxyflavone), the flavonoids from *Millingtonia hortensis* flower (Chulasiri, 1998). Hispidulin has been demonstrated to be a bronchodilator (Anulakanapakorn, Bunyapraphatsara, and Satayairvad, 1987), whereas hortensin has been possed anticancer properties (Hase *et al*, 1995). At the highest dose tested, 100 μg/plate, both compounds showed no mutagenicity and no cytotoxicity using the liquid preincubation method of the *Salmonella*/microsome test. These substances were antimutagens toward 2-aminoanthracene, aflatoxin B1 (in TA 98) and

dimethylnitrosamine (in TA 100); but neither substance inhibited the direct mutagenic activity of 2-(2-furyl)-3-(5-nitro-2-furyl) acryamide nor that of sodium azide in strains TA 98 and TA 100, respectively (Chulasiri, Bunyapraphatsara and Moongkarndi, 1992). Tansuwanwong *et al.*, 2006 studied that the aqueous extract from bark of *Millingtonia hortensis* had the ability to inhibit RKO human colon cancer cell growth and proliferation via the apoptosis pathway.

Figure 11. *Millingtonia hortensis* Linn. (Indian cork tree, ปีป)

2.2.9 *Rhinacanthus nasutus* ((Linn.) Kurz) (Thong pun chang, ทองพันชั่ง): ACANTHACEAE

It is a medicinal plant that is widely distributed in Southeast Asia, however it is known as "Thong pun chang" in Thailand (Figure 12). The plant is a small shrub, up to 1.5 m high; the stem is obtusely quadrangular, when young it is covered with fine, up curved hairs. Flowers are white, in short axillary clusters; densely appressed-pubescent (Farnsworth and Bunyapraphatsara, 1992).

Pharmacological Activity of Thong Pun Chang Flower

This plant is also commonly considered as a treatment for a number of disorders including cancer, fungal infections, eczema, pulmonary tuberculosis and herpes virus infections (Punturee *et al.*, 2005; Kernan *et al.*, 1997). In human peripheral blood mononuclear cells, water and ethanol extract of whole parts of *Rhinacanthus nasutus* significantly increased proliferation and the production of Interleukine-2 and tumor necrosis factor-α. (Punturee *et al.*, 2005). *Rhinacanthus nasutus* extracts could either increase or decrease nitric oxide production by macrophages and that these effects are predominantly mediated through an effect on tumor necrosis factor-α expression (Punturee, Wild and Vinitketkumnuen, 2004). The

ethanol extract of root and aqueous extract of leaves of *Rhinacanthus nasutus* and the active moiety Rhinacanthin C (chemically synthesized from *Rhinacanthus nasutus*) exhibited *in vitro* antiproliferative activity on PC-3 and T 24, which are human prostate and bladder carcinoma cell lines, respectively (Gotoh *et al.*, 2004). Moreover, the antitumour activity of Rhinacanthone, which isolated from the aerial parts of *Rhinacanthus nasutus*, has been evaluated against Dalton's ascitic lymphoma (DAL) in Swiss albino mice. When these rhinacanthone treated animals underwent intraperitoneal inoculation with DAL cells, tumour cell growth was found to be inhibited (Thirumurugan, Kavimani and Srivastava, 2000). In Thailand, the roots and leaves of *Rhinacanthus nasutus* are used for the treatment of cancer (Kongkathip *et al.*, 2004). In addition rhinacanthins-C,-N and –Q (three main naphthoquinone esters isolated from the roots of *Rhinacanthus nasutus*) induced apoptosis of human cervical carcinoma (HeLaS3) cells (Siripong *et al.*, 2006). There was no study of thong pun chang flower.

Figure 12. *Rhinacanthus nasutus* ((Linn.) Kurz) (Thong pun chang, ทองพันชั่ง)

2.2.10 *Punica granatum* Linn. (Pomegranate, ทับทิม): PUNICACEAE

Punica granatum, commonly known as pomegranate, is a small tree, belonging to the Punicaceae family (Figure 13). Pomegranate is grown mainly in Iran, India, America and in most Near and Far East countries. Flowers are showy, orange red, about 3 cm in diameter, 1-5 borned at branch tips. Fruit is globose berry, crowed by persistent calyx-lobes, having pericarp leathery filled with numerous seeds, which are surrounded by pink and red, transparent, juicy, acid, pleasant tasting pulp (Farnsworth and Bunyapraphatsara, 1992). It was consumed around the world as

edible fruit. In adition, pomegranate flower is consumed worldwide as Turkey and is in popularity as a beverage (Celik, Temur and Isik , 2008).

Pharmacological Activity of Pomegranate Flower

The plant possesses an immense therapeutic value. A number of biological activities such as antitumor (Afaq *et al.*, 2005), antibacterial (Prashanth, Asha and Amit, 2001), antidiarrhoeal (Das, *et al.*, 1999), antifungal (Dutta, Rahman and Das, 1998), antiulcer (Gharzouli *et al.,* 1999) have been reported with various extracts / constituents of different parts of this plant. In Thailand, hot water extract of dried root was taken orally as an anthelmintic. Hot water extract of dried fruit peel was taken orally for treatment of diarrhea and dysentery (Ross, 2005). The flowering part of *Punica granatum* has been recommended in *Unani* and *Ayurvedic* medicines as a remedy for diabetes (Li *et al*, 2005; Huang *et al*, 2005(a). Jafri *et al.* (2000) found that oral administration of its aqueous-ethanolic (50% v/v) extract of *Punica granatum* flower led to significant blood glucose lowering effect in normal, glucose-fed hyperglycemic and alloxan-induced diabetic rats. Other studies, oral administration of methanolic extract of *Punica granatum* markedly lowered plasma glucose levels in non-fasted Zucker diabetic fatty rats (a genetic model of obesity and type 2 diabetes), whereas it had little effect in the fasted animals, suggesting it affected postprandial hyperglycemia in type 2 diabetes. The extract was found to markedly inhibit the increase of plasma glucose levels after sucrose loading, but not after glucose loading in mice, and it had no effect on glucose levels in normal mice (Li *et al.*, 2005). In addition, pomegranate flower extract diminished cardiac fibrosis in Zucker diabetic fatty rats, a genetic animal model of type 2 diabetes and obesity (Huang *et al*, 2005b). Ethanolic extract of *Punica granatum* flowers showed 81.6 % antioxidant activity in DPPH model system. The extract also inhibited ·OH induced oxidation of lipids and proteins in vitro. The efficacy of extract was tested *in vivo* and it was found to exhibit a potent protective activity in acute oxidative tissue injury animal model: ferric nitrilotriacetate (Fe-NTA) induced hepatotoxicity in mice. Pretreatment with pomegranate flower extract at a dose regimen of 50-150 mg/kg body weight for a week significantly and dose dependently protected against Fe-NTA induced oxidative stress as well as hepatic injury (Kaur *et al.*, 2006). Polyphenol compound named pomegranatate, together with ellagic acid, 3,3′,4-tri-*O*-methylellagic acid, ethylbrevifolincarboxylate, ursolic and maslinic acids and daucosterol were isolated from ethanolic extract of the flowers of *Punica granatum* (Wang *et al.*, 2006). Celik,

Temur and Isik (2008) studied that while administration of subacute trichloroacetic acid promotes MDA (malonaldehyde) concentration fluctuates in the antioxidative systems and elevated tissue damage serum marker enzymes in rats model. The pomegranate flower beverage supplementation impart protection against carcinogenic chemical induced liver injury and oxidative stress.

Figure 13. *Punica granatum* Linn. (Pomegranate, ทับทิม)

2.3 Nitrite as a Converter for Direct-Acting Mutagens

Nitrate and nitrite occur in the diet from numerous different sources (Knight *et al.*, 1987). Vegetables are major sources of nitrate; nitrates alone are not toxic, which is converted to nitrite when such foods are stored at room temperature (Weisburgur and Raineri, 1975). The salts of nitrate and nitrite were used as a food additive for preservation due to antimicrobial properties. Particularly inhibition of the growth of *Clostridium botulinum* and their ability give a well color and taste (Buiatti *et al.*, 1990; Hoshiyama and Sasaba, 1992).

Various food produced in Asia were reported on their direct-acting mutagenicity after nitrite treatment. Kimchi, sun-dried fish, sun-dried squids, soy sauces, fish sauces, bean pastes and shrimp paste produced in Korea, the Philippines and Thailand showed direct-acting mutagenicity after nitrite treatment (Wakabayashi *et al.*, 1985). Palli (1996) indicated that salted/smoked and pickled/preserved foods (rich in salt, nitrites and preformed nitroso compounds) were associated with an increased risk of gastric cancer. Additionally, the extracts of raw and pickled vegetables and fruit, namely garlic, cabbage, shallot, mushroom, cucumber, ginger, Chinese mustard, bamboo shoot and mango were treated with nitrite in the absence of metabolic

activation. All of them exhibited direct-acting mutagenicity in *Salmonella* assay (Hankimhun, 1997).

The interaction of orally administered drugs with nitrite under mildly acidic conditions has been considered from a safety point of view of the drugs. Common drugs including aminopyrene (tertiary amines) react with nitrite to from dimethylnitrosamine or diakylnitrosamines (Lijinsky, Conard; and Van de Bogard., 1972; Lijinsky 1974). Phenolic drugs including bamethan, acetaminophen, and etilefrin also became mutagenic on treatment with nitrite under mildly acidic condition (Kikugawa, Kato, and Takeda, 1987, 1989; Ohta *et al.*, 1988). Therefore, several nitrosable mutagen precursors in foods taken by people in high-risk areas might be the etiological factor of gastric cancer, investigation must be continued to elucidate whether nitrosable compounds are involved in the development of human cancer, particularly of the stomach.

2.4 1-Aminopyrene-Nitrite Mutagenicity Model for Antimutagenicity Study

1-Aminopyrene is a derivative of 1-nitropyrene found in human gastrointestinal tract. Anaerobic bacteria metabolize 1-nitropyrene to 1- aminopyrene. 1-Nitropyrene is generally a product of incomplete combustion and is the predominant nitro-PAHs emitted in diesel engine exhaust, exhaust of kerosine heaters, petroleum gas burners and food products as a result of pyrolysis of fat in meat during barbecuing (Rosenkranz and Mermelstein, 1983; Handa *et al.*, 1983; Tokiwa, Nakagawa, and Horikawa, 1985; Kinouchi, Tsutsui, and Ohnishi, 1986 and Edenharder, Von Petersdorff, and Rauscher, 1993). The most primary route of potential human exposure to 1-nitropyrene is inhalation.

1-Aminopyrene was known to be non-mutagenic when it was tested without metabolic activation (Kinouchi *et al.*, 1986). Kato *et al.* (1991) demonstrated that1-aminopyrene treated with nitrite at pH 3.0 and 37 °C showed mutagenicity to *Salmonella typhimurium* strains TA 98 and TA 100 without metabolic activation. The resulted agreed with the work of Kangsadalampai, Butryee and Manoonphol (1996) which stated that nitrite-treated 1-aminopyrene exhibited stronger mutagenicity than the authentic aminopyrene towards *Salmonella typhimurium* strains TA 98 (frame-shift mutation) and TA 100 (base-pair substitution mutation), in the absence of metabolic activation. The mutation appear to be due to the presence of nitroreductase (IARC, 1989) and O-acetyltransferase (Mermelstein *et al.*, 1981) which are the two

activating systems presented in bacterial cells for nitrite-treated 1-aminopyrene (supposed to be 1-nitropyrene). Such enzymes metabolize 1-nitropyrene to be arylhydroxylamine, which is active to interact with DNA. Evidences had been shown that 1-Nitropyrene induced tumor in experimental animals (El-Bayoumy Hecht, and Hoffmann, 1983; Rosenkranz and Mermelstein, 1983; Busby *et al.*, 1994). Thus, the mutagenicity of 1-aminopyrene and nitrite in acid condition has been established as a model for antimutagenicity study of some chemical concerning the phenomenon occurred during stomach digestion.

2.5 The Salmonella Mutagenicity Test (Ames test)

The Ames Salmonella/microsome mutagenicity assay evolved over the years from the initial screening of a number of histidine mutants which led to the selection of mutants that were highly sensitive to reversion by a variety of chemical mutagens (Ames, 1971; Ames, Lee and Durston, 1973 (a); Levin, Yamasaki and Ames, 1982 (a); Levin *et al.*, 1982 (b); Maron and Ames, 1983). Because bacteria are unable to metabolize chemicals via cytochromes P450, as in mammals and other vertebrates, a key component for making the bacterial mutagenicity test useful was the inclusion of an exogenous mammalian metabolic activation system (Ames *et al.*, 1973; Malling, 1971). At the same time, the development of the plate incorporation assay protocol to replace spot test or liquid suspension procedures was a major contributing factor to the success of the Ames test because it made the test easier to perform and reduced its cost.

2.5.1 Screening of Histidine Mutants

Studies performed to identify and map the genes responsible for histidine biosynthesis produced a large number of spontaneous, radiation, and chemical-induced histidine mutants of *Salmonella typhimurium* LT-2 (Whitfield, Martin and Ames, 1966; Hartman *et al.*, 1971). Some of the mutants contained single base changes (base-pair substitution mutants), and others contained additions or deletions of one or more bases (frameshift mutants). It was later realized that some of these mutant strains could be used to identify and characterize mutagenic chemicals by their ability to revert to wild-type (histidine-independence) in the presence of mutagens. In 1966, Ames and Whitfield proposed a set of histidine mutant strains for screening chemicals for mutagens using a spot test procedure. The spot test consists of applying a small amount of the test chemical directly to the center of a selective agar medium

plate seeded with the test organism. As the chemical diffuses into the agar a concentration gradient is formed. A mutagenic chemical will give rise to a ring of revertant colonies surrounding the area where the chemical was applied.

2.5.2 Development of the Plate Incorporation Assay

In 1973, Ames *et al.* (1973 a and b) developed the plate incorporation assay procedure which is more sensitive and quantitative than the spot test. The procedure consists of adding the buffer or S-9 mix, the histidine dependent bacteria (about 10^8) and test chemical to 2 ml of top agar containing biotin and a trace amount of histidine (0.05 mM each). The mixture is then gently mixed and poured on glucose minimal agar plates. When the top agar has solidified the plates are incubated in an inverted position in a 37 °C incubator for 48 h at which time the histidine revertant colonies are counted. The small amount of histidine allows all the plated bacteria to undergo a few cell divisions; in many cases, this growth is essential for mutagenesis to occur. The histidine independence (His^+) revertants are easily scored as colonies against the slight background growth. The number of spontaneously induced revertant colonies is relatively constant for each strain. However, when a mutagen is added to the plate, the number of revertant colonies per plate is increased, usually in a dose related manner as depicted in Figure 14.

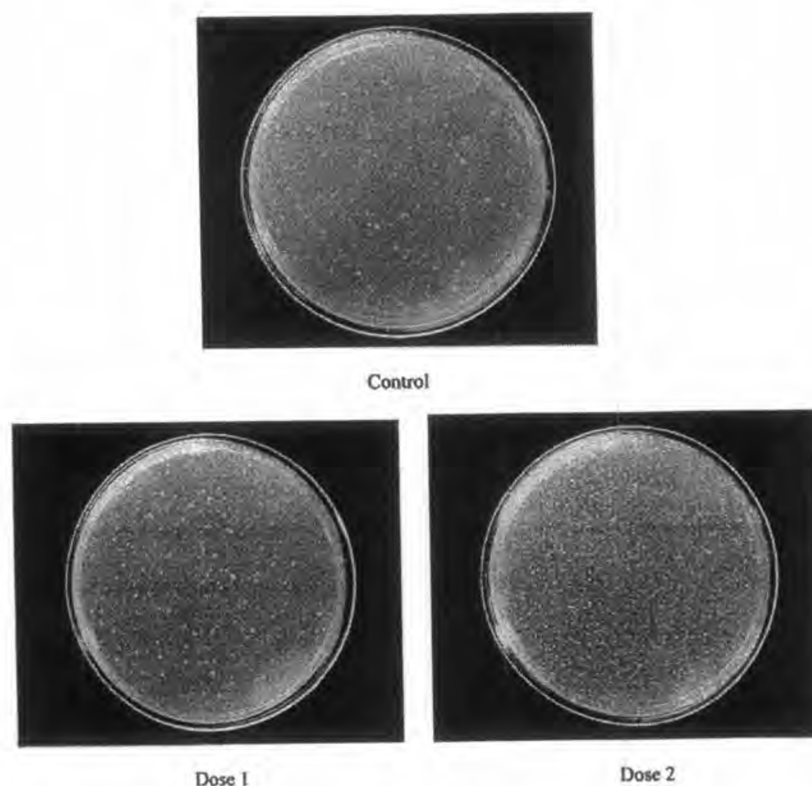

Figure 14. Mutagenic dose response with strain TA 100 and sodium azide. Control: spontaneous revertants; dose 1: 2.5 μg/plates; dose 2: 5 μg/plates (Mortelmans and Zeiger, 2000).

2.5.3 Metabolic activation systems

Some carcinogenic chemicals, such aromatic amines or polycyclic aromatic hydrocarbons, are biologically inactive unless they are metabolized to active form. In human and lower animals, the cytochrome-based P450 metabolic oxidation system, which is present mainly in the liver and to a lesser extent in the lung and kidneys, is capable of metabolizing a large number of these chemicals to DNA-reactive, electrophilic forms. Some of the intermediate metabolites are potent mutagens in the Ames *Salmonella* assay. Since bacteria do not have this metabolic capability, an exogenous mammalian organ activation system needs to be added to the petri plate together with the test chemical and the bacteria. For this purpose, a rodent metabolic activation system was introduced into the test system (Smith, 1966; Malling, 1971; Miller and Miller, 1971; Garner, Miller and Miller, 1972; Ames, Lee and Durston, 1973). The metabolic activation system usually consists of a 9000xg supernatant fraction of a rat liver homogenate (S-9 microsomal fraction), which is delivered to the test system in the presence of NADP and cofactors for NADPH-supported oxidation

(S-9 mix) (Maron and Ames, 1983). To increase the level of metabolizing enzymes, the animals are pretreated with the mixed-function oxidase inducer Aroclor 1254. Other inducers, such as Phenobarbital and β–naphthoflavone, can also be used.

The metabolic activation system can also consist of a reductive enzyme system for classes of chemicals containing azo and diazo bonds. Reduction of chemicals substances can occur in mammals, including humans, by anaerobic intestinal microflora, and very likely by mammalian reductases in the intestinal wall or in the liver. Two types of reductive in vitro metabolic activation systems have generally been used, those based on a liver homogenate supplemented with FMN (Prival and Mitchell, 1982; Prival *et al.*, 1984), and those that are based on rat intestinal microflora preparations (Reid *et al.*, 1983; Reid *et al.*, 1984a and b).

2.5.4 The Salmonella Tester Strains

The genotype of the commonly used Salmonella tester strains are listed in Table 2. All strains are histidine dependent by virtue of a mutation in the histidine operon. Additional mutations/genetic alterations that have made the tester strains more sensitive to chemical mutagens are listed below.

Table 2 Genotype of the most commonly used Salmonella tester strains (Mortelmans and Zeiger, 2000).

Mutation (strain)	*bio chlD uvrB gal*	Lipolysaccharide defect	Plasmid
hisG46			
TA 1535	Deletion mutation	*rfa*	No plasmid
TA 100	Deletion mutation	*rfa*	pKM101
hisD3052			
TA 1538	Deletion mutation	*rfa*	No plasmid
TA 98	Deletion mutation	*rfa*	pKM101
hisC3076			
TA 1537	Deletion mutation	*rfa*	No plasmid
hisD6610	Deletion mutation	*rfa*	pKM101
hisO1242			
TA 97			
hisG428			
TA 104	Deletion mutation	*rfa*	No plasmid
TA 102	Wild type	*rfa*	pKM101,pAQ1

1. A deletion mutations through the *uvrB-bio* genes in all strains, except TA 102. The *uvrB* deletion mutation eliminates the accurate excision repair mechanism, thereby allowing more DNA lesions to be repaired by the error-prone DNA repair mechanism. The deletion through the biotin gene makes the bacteria biotin dependent. (Ames *et al.*, 1973a)

2. A mutation (*rfa*) in all strains that leads to a defective lipolysaccharide layer that coats the bacterial surface, making the bacteria more permeable to bulky chemicals (Ames *et al.*, 1973a).

3. Introduction of plasmid pKM 101 in strains TA 1535 and TA 1538 resulting in the corresponding isogenic strains TA 100 and TA 98(Ames, McCan and Yamasaki, 1975) and in strains TA 97 (Levin *et al.*, 1982a) and TA 102 and TA 104 (Levin *et al.*, 1982b). Plasmid pKM 101 enhances chemical and UV-induced mutagenesis via an increase in the error-prone recombinational DNA repair pathway (McCann *et al.*, 1975b; Walker and Dobson, 1979; Shanabruch and Walker, 1980). The plasmid confers ampicillin resistance, which is a convenient marker to detect the presence of the plasmid (Mortelmans and Stocker, 1979).

4. Insertion of the mutation *hisG428* on the multicopy plasmid pAQ1 which was introduced in strain TA 102 with the aim of amplifying the number of target sites. To enhance the ability of this strain to detect DNA cross-linking agents, the *uvrB* gene was retained making the bacterium DNA repair proficient (Levin *et al.*, 1982b).

2.5.5 Validation Studies

Over the years, many validation studies have been performed to determine the reproducibility of test results on an intra- and inter- laboratory level (Purchase *et al.*, 1978; Venitt and Crofton-Sleigh, 1981; Dunkel *et al.*, 1984; Margolin *et al.*, 1984; Dunkel *et al.*, 1985; Zeiger, 1985; Piegorsch and Zeiger, 1991). In addition, many studies have been performed to determine the sensitivity and correlation of the Ames test with animal carcinogenicity studies. It has indeed been established that there is a high predictivity of a positive mutagenic response in the Ames test for rodent carcinogenicity (Tennant *et al.*, 1987; McCann *et al.*, 1975a; Ames and McCann, 1976; Sugimura *et al.*, 1976; Zeiger, 1997), ranging from 90% (McCann *et al.*, 1975a) to 77% (Zeiger, 1998), the primary differences being the chemical composition of the compiled database. The test therefore is in many instances used as a first screen to determine the mutagenic potential of new chemicals and drugs.

2.5.6 Spontaneous Control Values

Each tester strains has a characteristic spontaneous mutant frequency. There is usually some day-to-day and laboratory-to-laboratory variation in the number of spontaneous revertant colonies. Choice of solvent may also affect the spontaneous mutant frequency (Maron *et al.*, 1981). Table 3 presents a range of spontaneous histidine revertant (negative control) control values per plate with and without metabolic activation. The values obtained in the presence of a metabolic activation system includes both rat and hamster liver S-9. The spontaneous values presented for S-9 were from 10% S-9 in the S-9 mix. Some of the strains (e.g., TA 97, TA 102, TA 104) are highly sensitive to S-9 concentrations and their spontaneous reversion values will increase with the S-9 concentration.

Table 3 Spontaneous revertant control values (Mortelmans and Zeiger, 2000).

Strain	Number of revertants	
	Without S-9	With S-9
TA 97	75-200	100-200
TA 98	20-50	20-50
TA 100	75-200	75-200
TA 102	100-300	200-400
TA 104	200-300	300-400
TA 1535	5-20	5-20
TA 1537	5-20	5-20
TA 1538	5-20	5-20

2.5.7 Toxicity Determination

Toxicity determination in the Ames Salmonella test requires the evaluation of characteristics of the final population on the glucose minimal agar plate after the 48 h incubation instead of a quantitative determination. These characteristics are:

1. Thinning of the background lawn which may be accompanied by a decrease in the number of revertant colonies

2. absence of background lawn (i.e., complete absence of growth)

3. presence of pinpoint non-revertant colonies (generally in conjunction with an absence of background lawn)

Microscopic (40x) examination of the background lawn in the absence of toxicity will reveal the presence of densly packed microcolonies which form a uniform, though somewhat granular thin film. In such cases, all the plated histidine-dependent bacteria were able to undergo six to eight cell divisions. However, when a chemical is toxic there may be "thinning" or complete absence of the background lawn compared to the negative or solvent control. Partial toxicity of the chemical will give rise to thinning since not all the plated bacteria were killed or had their growth inhibited. In this case, the surviving bacteria still form microcolonies but they are not densely packed and may appear as single sparsely spaced microcolonies which results in the "thining" effects; those colonies will not be visible to the naked eye. A decrease in the number of revertant colonies to levels below the spontaneous reversion level may on occasion be seen with thinning. A complete absence of background lawn indicates a high level of toxicity with the inability of the bacteria to grow and form a lawn. Such a toxic dose should not be used.

Occasionally numerous small non-revertant colonies are present on the plate. The colonies are referred to as "pinpoint colonies" and consist of histidine-dependent bacteria that survived high chemical toxicity. These colonies are readily visible by the naked eye and may be mistaken for revertant colonies. Microscopic inspection of the plates will, however, reveal that there is a total absence of background lawn. The pinpoint colonies arise due to the fact that the high level of toxicity resulted in more histidine being available to the surviving His- bacteria on a per cell basis. Therefore, these bacteria can undergo additional cell divisions until the depletion of the histidine. A preliminary toxicity experiment is usually performed to determine the top dose that can be tested.

2.6 Somatic Mutation and Recombination Test (SMART)

The somatic mutation and recombination test (SMART) is one of the first assay systems used to test chemical and physical agents for recombinogenic effects and provides a number of methodological advantages to detect genotoxicity of chemical substances (Hiraizumi, 1979). The SMART in *Drosophila melanogaster* has been designed to detect genetic damage in a sensitive, rapid and inexpensive way. It is an *in vivo* system that uses a eukaryotic organism with metabolic machinery similar to that found in mammalian cells (Graf *et al.*, 1984 and 1989; Vogel and Zijlstra, 1987). Several advantages of *Drosophila melanogaster* as a test organism for detection of

chemicals with genotoxic activity have been enumerated. The main points are as following. It has a short life cycle (10 days at 25°C). It has easily detectable genetically controlled morphological characters. Large numbers of mutants and genetically characterized strains are available. Culture media are inexpensive and allow the breeding of large numbers of animals using simple facilities. And it is capable of activating enzymatically promutagens and procarcinogens *in vivo* (Sarıkaya and Çakır, 2005).

This assay is based on induced loss of the heterozygosity, which may occur through various mechanisms, such as point mutations, deletions, certain types of chromosome aberrations as well as mitotic recombination and gene conversion (Graf *et al.*, 1984). It is based on the treatment of larvae during the embryogenesis, the imaginal disc cells proliferate mitotically and many genetic events such as point mutation, deletion, somatic recombination and non-disjunction can be determined on the wing of adult flies (Würgler and Vogel, 1986). If a genetic alteration occurs in one cell of the imaginal disc during mitotic proliferation, it will form a clone of mutant cells expressing the phenotype regulated by the specific genetic markers. The use of improved high-bioactivation (HB) strains of *Drosophila melanogaster*, which are characterized by increased cytochrome P450-dependent bioactivation capacity, facilitates the detection of promutagens and procarcinogens of different chemical classes (Graf and Singer, 1989; Graf and Van Schaik, 1992).

The SMART assay is also well suited to determine the antimutagenicity of pure chemicals or mixtures (Negishi *et al.*, 1989; Graf *et al.*, 1989). The wing spot test has been used for genotoxicity studies with several kinds of beverages, such as different types of coffees, various herbal teas as well as wines and brandy (Graf and Van Schaik, 1992). Negishi *et al.* (1989) reported inhibitory effect of chlorophyllin on the genotoxicity of Trp-P-2 (3-amino-1-methyl-*5H*-pyrido[4,3-*b*]indole) in the wing spot test. Katz (1989) showed that sodium thiosulfate was an efficient inhibitor of cisplatin-induced mutagenesis using the SMART. With respect to the antigenotoxicity of complex mixtures, Abraham and Graf (1996) indicated that instant coffee demonstrated protective effects against series of known mutagens and carcinogens. Several researchers published some papers on the modulation of genotoxicity in the wing somatic cells by various modulating agents such as enzyme inhibitors or inducers (Cederberg and Ramel, 1989; Romert Magnusson, and Ramel, 1990).

2.6.1 Wing Spot Test in *Drosophila* The wing spot test makes use of the recessive markers multiple wing hair (*mwh*) and flare (*flr*^{3}) which alter the phenotypic expression of the hairs on the wing blade (Graf *et al.*, 1984 and 1989; Szabad *et al.*, 1983). The two wing hair markers are both located on the left arm of chromosome 3. The appearance of multiple wing hairs (*mwh*, 3-0.0) is a recessive, homozygously viable mutation and produces multiple trichomes per cell instead of the normally unique trichome. The second marker, flare (*flr*^{3}, 3-39.0) is a recessive mutation that produces malformed wing hairs that have the shape of a flare. All three mutant alleles of *flr* are recessive zygotic lethals. However, homozygous cells in the wing imaginal discs are viable and lead to mutant wing cells. The *flr*^{3} allele is kept over a balancer chromosome carrying multiple inversions and a dominant marker that is a homozygous lethal (*flr*^{3}/*TM3*, *Bd*^{s}: Third Multiple 3, Beaded-Serrate).

In all the experimental series analyzed, the occurrence of the various types of spots was as follows: most frequent were single spots expressing the *mwh* phenotype, less frequent twin spots with both a recombination sub-clone and quite rare single spots with the *flr*^{3} phenotype (Lindsley and Zimm, 1992). Different types of wing hair mutations are shown in Figure 15.

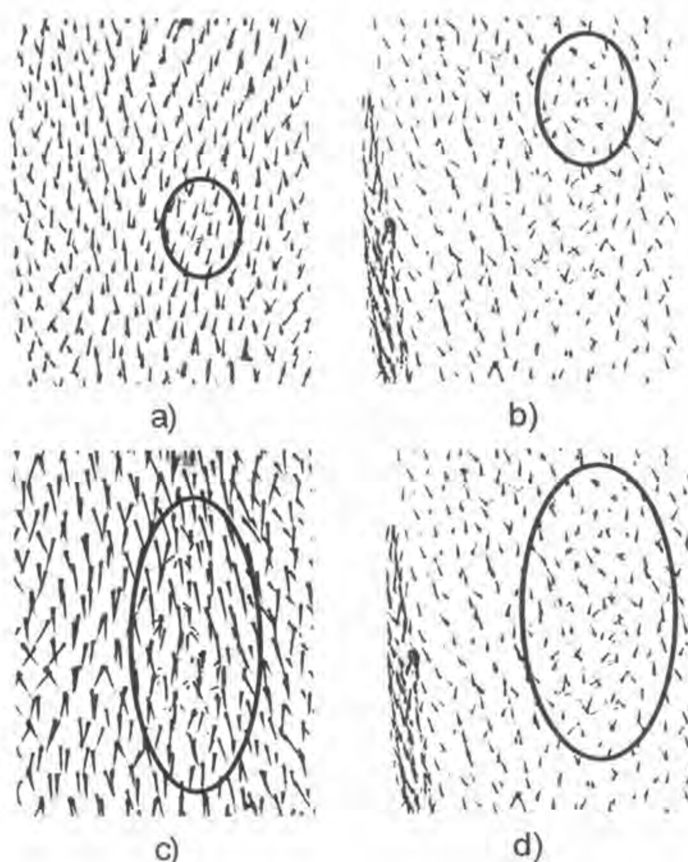

Figure 15. Marker mutations of wing surface to show clone of cuticle secreted by cells homozygous for multiple wing hairs, a) small single spots of *mwh* on wing, b) large single spots of flare on wing, c) large single spots of *mwh* on wing, d) twin spots (By courtesy of Kaew Kangsadalampai).

Several mechanisms lead to genetically marked clones (Figure 16). An important possibility is a mitotic recombination event between two non-sister chromatids. Twin spots are expected if recombination occurs between flr^3 and the centromere (Becker, 1976). A recombination event between *mwh* and flr^3 may result in a *mwh* single spot. If both types of recombination events (one between flr^3 and the centromere, a second between *mwh* and flr^3) take place within the same cell, a flr^3 single spot may result. Non disjunctional or other loses of the chromosomes carrying the wild type allele represents another mechanism that may lead to single spots. Mitotic recombination in the chromosome section between the centromere (spindle fiber attachment site) and the marker flr^3 leads to two daughter cells, one homozygous for *mwh*, the other homozygous for flr^3. Clonal expansion to these two cells will be recognizable on the wing blade from the two multicellular adjacent clones, one exhibiting the *mwh* phenotype (multiple hairs), the other the flr^3 phenotype (misshape hairs). On the other hand, the origin of "single spots", showing either the *mwh* or the flr^3 phenotype (mainly of the *mwh* phenotype, rarely also of the flr^3 phenotype), cannot be clearly determined. Multiple wing hairs single spots may result from a recombination event occurring in the chromosome segment between the two marker genes. In addition, a gene mutation or deletion of the mwh^+ gene will result in a *mwh* single spot. A flr^3 single spot may either result from a gene mutation or a deletion of the flr^3 gene, or from a rare double recombination with one recombination event to the left and the other event to the right of the flr^3 locus (Würgler, Graf and Frolich, 1991).

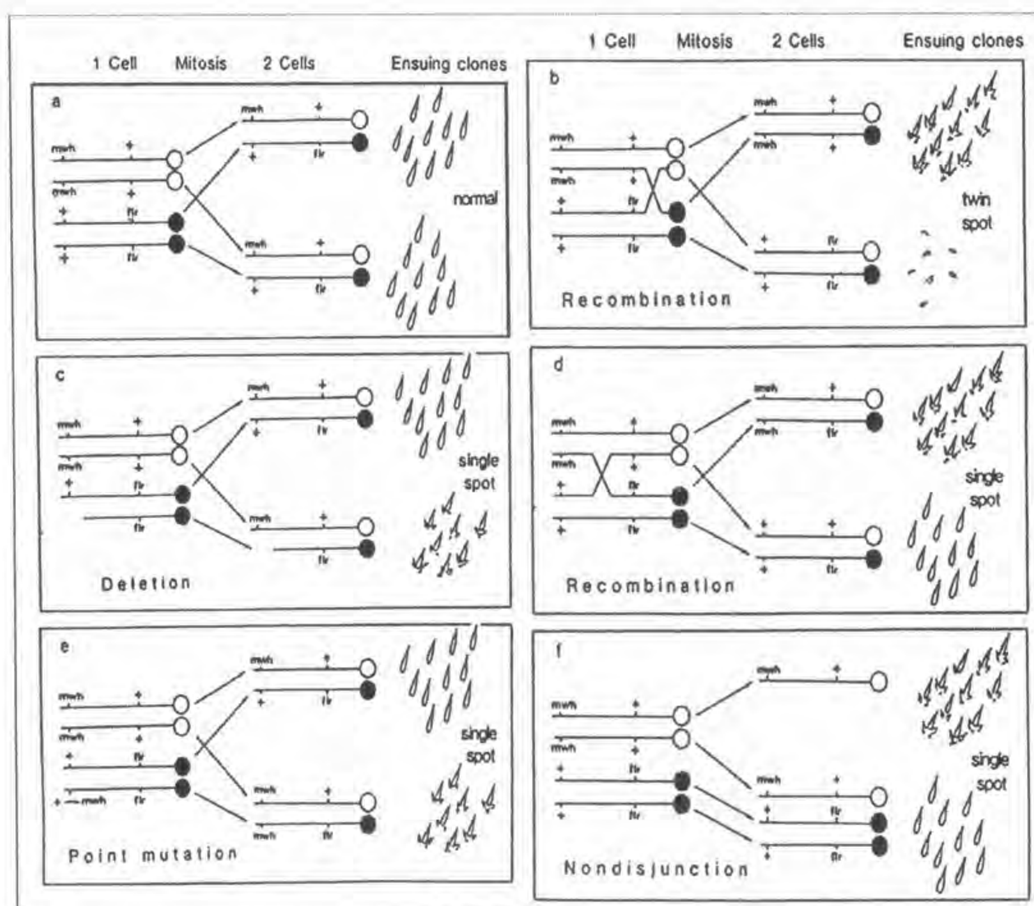

Figure 16. Genetics schemes illustrating various ways of spot formation in the somatic mutation and recombination test with the wing cell markers multiple wing hairs (*mwh*) and flare (*flr*^{3})(a). Twin spots are obtained by recombination proximal to the *flr*^{3} marker (b), while more distal recombination produces *mwh* single spots only (d). Deficiencies (c), point mutations (e) and nondisjunction events (f) give rise to *mwh* single spots or in analogous ways to *flr*^{3} single spots (not illustrated) (Graf *et al.*, 1984).

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2.6.2 Standard Mutagen for Mutagenicity of SMART Urethane ($NH_2COOCH_2CH_3$), also known as ethyl carbamate, is the ethyl ester of carbamic acid (NH_2COOH). Urethane may occur as a colorless, odorless crystal white, granular powder. It is slightly soluble in olive oil and soluble in water, ether, glycerol, chloroform and ethyl ether. The major source of human exposure to urethane is from fermented food products (bread, yogurt and cheese) and alcoholic beverages (white wine and beer) (IARC, 1974; Ough, 1976; Miller and Miller, 1983; Canas *et al.*, 1989). Urethane is used as both an animal anesthetic (Kotanidou *et al.*, 1996; Norlen *et al.*, 2000) and an industrial chemical (Crout, 1976). Furthermore, it was shown to induce genotoxicity in *Drosophila melanogaster* (Zimmerli and Schlatter, 1991). Urethane is generally used as positive standard toxicants in evaluation genotoxicity of the unknown compounds in SMART (Abraham and Graf, 1996).

2.6.2.1 Metabolic Activation and Detoxification of Urethane

Urethane was found to induce point mutation, gene conversion, intrachromosomal recombination, chromosomal aberrations and sister chromatid exchanges in yeast, plant systems and mammalian cells (Schlatter and Luitz, 1990). This chemical requires metabolic activation to express its mutagenic activity (Frölich and Würgler, 1990b). Urethane is metabolized by at least three pathways (Salmon, Landy and Zeise 1991; Park *et al.*, 1993) as shown in Figure 17. In rodents, more than 90 % of an administered dose of urethane is hydrolyzed to ethanol, ammonia and carbon dioxide by liver microsomal esterases and amidase (Mirvish, 1968; Park *et al.*, 1993). This pathway is probably one for detoxification (IARC, 1974). Approximately 0.1% of urethane is reversibly converted by cytochrome P450 subtype 2E1 (CYP2E1) to 2-hydroxyethyl carbamate (Guengerich and Kim, 1991), a compound that is inactive as a carcinogen (Berenblum *et al.*, 1959), to *N*-hydroxyethyl carbamate (Boyland and Nery, 1965; Nery, 1968), a compound that is less carcinogenic than urethane (Mirvish, 1968). Less than 0.5% of urethane is metabolized by CYP2E1 to vinyl carbamate and the metabolite is more potent than its parent compound in its carcinogenicity. Vinyl carbamate, in turn, is converted by epoxidation to the putative ultimate carcinogen vinyl carbamate epoxide (Miller and Miller, 1983; Guengerich and Kim, 1991; Guengerich, Kim, and Iwasaki, 1991). Vinyl carbamate epoxide can covalently bind to DNA, RNA and proteins to form adducts and the initiation of tumorigenesis (Dahl Miller, and Miller 1978; Miller and Miller, 1983; Leithauser *et*

al., 1990). The schematic structures of urethane and its metabolites are shown in Figure 18.

ACTIVATION INACTIVATION

$H_3C\text{-}CH_2\text{-}O\text{-}C(=O)\text{-}NH_2$ [a]
Ethyl carbamate (urethane)

H_2O, Amidases/Esterases → $H_3C\text{-}CH_2\text{-}OH$ + CO_2 + NH_3
cytochrome P-450[b], O_2, NADPH → $HO\text{-}CH_2\text{-}CH_2\text{-}O\text{-}C(=O)\text{-}NH_2$
cytochrome P-450[b], O_2, NADPH → $H_3C\text{-}CH_2\text{-}O\text{-}C(=O)\text{-}NH\text{-}OH$ (in vivo ←)

cytochrome P-450[b], O_2, NADPH

$H_2C{=}CH\text{-}O\text{-}C(=O)\text{-}NH_2$ [a]
Vinyl carbamate

H_2O, Amidases/Esterases → $H_3C\text{-}CHO$ + CO_2 + NH_3

cytochrome P-450[b], O_2, NADPH

$H_2C(O)CH\text{-}O\text{-}C(=O)\text{-}NH_2$ [c]
Vinyl carbamate epoxide

H_2O, pH 7.4, Phosphate → $HO\text{-}CH_2\text{-}CHO$ + CO_2 + NH_3
+ 3 other non-mutagenic reducing compounds
0.154 M Cl ion, pH 7.4, Phosphate → $ClCH_2\text{-}CHO$ + CO_2 + NH_3
+ 3 other reducing compounds
Epoxide hydrolases, H_2O → $HO\text{-}CH_2\text{-}CHO$ + CO_2 + NH_3
+ other products ?
GSH-S-transferases, GSH → $G\text{-}S\text{-}CH_2\text{-}CHO$
$(HOOC\text{-}CH_2)_2S$
$HO\text{-}(CH_2)_2\text{-}S\text{-}CH_2\text{-}CH(NH\text{-}C(=O)\text{-}CH_3)\text{-}COOH$

DNA adducts in protooncogenes

Initiation of carcinogenesis

Figure 17. Known and probable activation and inactivation pathways of metabolism of urethane (ethyl carbamate), vinyl carbamate and vinyl carbamate epoxide, (a) Mouse liver microsomes + ethyl carbamate or vinyl carbamate + adenosine → 1,N^6-ethenoadenosine, (b) Human liver microsomal cytochrome P450 IIE1, (c) Vinyl carbamate epoxide + adenosine → 1, N^6-ethenoadenosine. GSH = glutathione (Park *etal.*,1993).

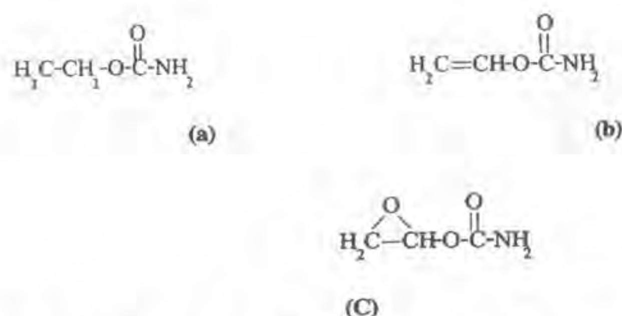

Figure 18. Schematic structures of urethane and its metabolites (a) Urethane (ethyl carbamate); (b) Vinyl carbamate; (c) Vinyl carbamate epoxide (Park *et al.*, 1993).

2.6.2.2 Mutagenicity of Urethane

Urethane was discovered as carcinogen in 1943 (Nettleship *et al.*, 1943). Many reports were published concerning the mutagenicity of urethane in a wide range of organism (Field and Lang, 1988). It was shown to induce a multiple carcinogen in various organs and animal species (Benson and Beland, 1997). In rodents, urethane is a multi-potential animal carcinogen, producing lung tumors, lymphomas, hepatomas, melanomas and vascular tumors (Mirvish, 1968; IARC, 1974; Mori *et al.*, 2000). Park *et al.* (1993) showed that vinyl carbamate and the oxide metabolite produced guanine adducts in both mouse and rat liver DNA. In bacterial mutagen test systems urethane is usually regarded as a non-genotoxic agent. However, in *Salmonella typhimurium* in the presence of P450-mediated activation, urethane was reported to be mutagenic (Anderson and Styles, 1978). In mutagenicity tests using eukaryotic cells, positive and negative findings were about equal in frequency. It seemed that positive results were obtained only under conditions of appropriate metabolic activation. Urethane was genotoxic in the somatic mutation and recombination test in *Drosophila melanogaster* (number and shape of wing hairs after treatment of larvae), in a standard strain and in a modified strain (*ORR*) in which genetic control of cytochrome P450 dependent enzyme systems were altered (constitutively increased P450 enzyme activities) (Frölich and Würgler, 1988 and 1990a). The effects were dose-dependent and the modified strain (*ORR*) is more sensitive to urethane by about one order of magnitude than the standard strain. It was suggested that the P450 enzyme system be involved in the activation of urethane. The frequencies of spots per wing in high bioactivation cross were higher than that in standard cross (Frölich and Würgler, 1990a). This might result from the constitutive expression of the enzymes required for the transformation of urethane into ultimate genotoxic metabolites. In addition, urethane

gas induced a significant increase of X-linked recessive lethal mutations in the germ cells of *Drosophila melanogaster* (Nomura and Kurokawa, 1997). Urethane was classified as possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer, but evidence of urethane carcinogenicity in humans was considered inadequate (IARC, 1974).

2.6.2.3 Modification of the Mutagenicity of Urethane

The major detoxification pathway of urethane is through hydrolysis to ethanol, ammonia and carbon dioxide (IARC, 1974), a reaction mediated by CYP2E1 (Hoffler *et al.*, 2003). In rats, mice and humans, CYP2E1 is induced five to 20-fold by ethanol (Lieber, 1988 and 1990; Kurata *et al.*, 1991; Ingelman-Sundberg *et al.*, 1993), which suggests that chronic ethanol exposure could increase the oxidation of urethane to its epoxide derivative. On the other hand, ethanol has been reported to decrease the metabolism of urethane, presumably by acting as a competitive substrate (Waddell, Marlowe, and Pierce, 1987, Yamamoto *et al.*, 1988). Kristiansen, Clemmensen and Meyer (1990) exposed adult female A/Ph mice to 0, 200, 500, or 1000 ppm urethane simultaneously with 0%, 5%, 10%, or 20% ethanol in drinking water for 12 weeks. All mice treated with urethane developed lung adenomas with a dose-dependent manner. In addition, Mirvish (1968) reported that degradation of urethane was inhibited up to 90% by blocking esterase activity, which indicated that ethanol might be formed in near equimolar amounts to the administered urethane dose. It was also shown whether the ethanol thus formed and modulated the further metabolism of urethane. Kurata *et al.* (1991) demonstrated that acetone was a very potent, acute inhibitor of the *in vivo* metabolism of urethane when metabolically derived from 2-propanol. Conversely, pretreatment using acetone for 24 and 48 h before urethane administration accelerated the clearance of urethane, indicating that enzyme metabolizing urethane was induced by acetone.

Kemper, Myers, and Hurst (1995) investigated the role of glutathione in protection against vinyl carbamate epoxide-mediated adduct formation and the involvement of glutathione-*S*-transferase (GST) in detoxification of vinyl carbamate epoxide. They reported that glutathione inhibited formation of ethenoadenosine in a concentration-dependent manner ranging from 1 to 8 mM. This effect was significantly enhanced by addition of rat liver GST. In addition, De flora *et al.* (1986) reported that *N*-acetylcysteine (NAC), a precursor of intracellular glutathione, efficiently prevented the induction of lung tumors in Swiss albino mice, when

supplemented to the diet both before and after and i.p. injection of the carcinogen urethane. Irrespective of urethane administration, NAC also significantly enhanced GST activity in liver preparations of the same animals. Investigation on the change in GST activity in relation to the observed *in vivo* antigenotoxicity of fresh vegetables, spices, tea and coffee was done by Abraham, Singh and Kesavan (1998). This experiment showed that treatment with urethane alone resulted in inhibition of GST activity. Co-administration of urethane with extracts of vegetables, coffee and spices resulted in dosed-related attenuation of the inhibitory effect of urethane on GST activity. However, tea had no effect on inhibition of GST activity by urethane. Hence and association between antigenotoxicity and GST activity could not be established. Furthermore, Abraham and Graf (1996) investigated the protective effects of coffee against somatic mutation and mitotic recombination induced by urethane were evaluated in the standard (ST) and high bioactivation (HB) crosses of the wing spot test in *Drosophila melanogaster*. The results showed high sensitivity of the HB cross to urethane. Co-administration of instant coffee was effective in exerting significant dose-related inhibitory effects on the genotoxicity of urethane in the ST and the genetically susceptible HB cross. Pretreatment of 2-day old HB larvae with coffee for 24 h followed by treatment with urethane was also effective in significantly reducing the induction of mutation and recombination. The magnitude of the protective effects of coffee against the genotoxin (urethane) was independent of the genotype of the larvae used for treatment.

A dose-dependent increase in the genotoxic activity of urethane was observed in SMART (Frölich and Würgler, 1990b). The frequency of induction of mutation in the modification strain with increased cytochrome P450 enzyme activities was increased by about one order of magnitude compared with the standard strain. The frequencies of spots per wing in high bioactivation cross were higher than that in standard cross (Frölich and Würgler, 1990a). This might result from the constitutive expression of the enzymes required for the transformation of urethane into ultimate genotoxic metabolites.

2.7 Brine Shrimp Bioassays

The adult brine shrimp artemia is an extremely well known animal because of its importance as a food source for fish and crustaceans raised in home aquariums, aqua culture systems, and in laboratories. The common brine shrimp (artemia) is in the

phylum Arthropoda, class Crustaceae (Pelka *et al.*, 2000). The egg of brine shrimp, *Artemia salina* Leach, are readily available at low cost in pet shops as a food for tropical fish, and they remain viable for years in the dry state. Upon being placed in a brine solution, the eggs hatch within 48 hours, providing large number of larvae (nauplii). Brine shrimp assay is considered a useful tool for preliminary assessment of toxicity and it has been used for the detection of fungal toxins, plant extract toxicity, heavy metals, pesticides and cytotoxicity testing of dental materials (Pelka *et al.*, 2000; Krishnaraju *et al.*, 2005). It has the advantages of being rapid (24 hours following introduction of shrimp), inexpensive, and simple (no aseptic techniques are required). The procedure determines LC_{50} values in µg/ml of active compounds and extracts in the brine medium. Activities of a broad range of known active compounds are manifested as toxicity to the shrimp (Meyer *et al.*, 1982).

2.8 Antioxidant Assays

A wide range of methods are currently used to assess antioxidant capacity (Halliwell *et al.*, 1995), including measurement the prevention of oxidative damage to biomolecules such as lipids or DNA and methods assessing radical scavenging. Both *in vivo* and *in vitro* assays are used and all methods have their own advantages and limitations. Simple scavenging assays, such as the TRAP (total reactive antioxidant potential or total radical-trapping antioxidant parameter) and the TEAC (Trolox equivalent antioxidant capacity) assay, have gained popularity because of high-throughput screening on potential antioxidant capacity. Such methods are used to assess antioxidant capacity of biological matrices, such as plasma, as well as single compounds, food components, or food extracts.

2.8.1 2, 2′-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

DPPH assay is a common employed assay in antioxidant studies and offers a rapid technique in which to screen the radical scavenging activity of pure synthetic compounds, crude plant extracts and foods. The reaction of DPPH was used to compare the radical scavenging activity of a compound with Trolox, a water soluble vitamin E analogue (Van den Berg *et al.*, 1999; Pietta, Simonetti, and Mauri, 1988). 2,2′-Diphenyl-1-picrylhydrazyl (DPPH), also known as 1,1-diphenyl-2-picrylhydrazyl or R,R-diphenyl-*â*-picrylhydrazyl, is a free radical used for assessing antioxidant activity. Reduction of DPPH by an antioxidant or by a radical species results in a loss

of absorbance at 520 nm. Thus, the degree of discoloration of the solution indicates the scavenging efficiency of the added substance (Fukumoto and Mazza, 2000).

2.8.2 Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was carried out by the method of Benzie and Strain (1996) with minor modification. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}- TPTZ). The reaction is nonspecific, and any half-reaction which has a less-positive redox potential, under reaction condition, than the Fe^{3+}/Fe^{2+}-TPTZ half-reaction will drive Fe^{3+}-TPTZ reduction. Test conditions favor reduction of the complex and, thereby, color development, provided that a reductant (antioxidant) is present. Ferrozine (Stookey, 1970), a compound closely related to TPTZ, has been widely used, with excess ascorbic acid, to measure iron. In the FRAP assay, excess Fe^{3+} is used, and the rate-limiting factor of Fe^{2+}-TPTZ, and hence color, formation is the reducing ability of the sample.

2.8.3 Determination of Total Phenolic Contents

The total phenolic contents was determined according to the method described by Swain and Hillis (1959), Naczk and Shahidi (1989), Amarowicz *et al.* (2004) with minor modification. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Javanmardi *et al.*, 2003). Total phenolic content was estimated using the Folin-Ciocalteu colorimetric method. Briefly, the appropriate dilutions of the extracts were oxidized with Folin-Ciocalteu reagent and then the reaction was neutralized with saturated sodium carbonate. The absorbance of the resulting blue color was measured with a spectrophotometer after incubation. Quantification was done on the basis of the standard curve of gallic acid. Results were expressed as gallic acid equivalent (GAE) (Cai *et al.*, 2004).