CHAPTER II

LITERATURE REVIEW

2.1 Nitrite and Gastric Cancer

Nitrate and nitrite have been used for centuries in curing and preserving meats and fish and in the manufacture of certain cheeses (Binkerd and Kolari, 1975). Being added to these foods, nitrite has at least three functions. Firstly, it contributes to the favour; this may be due to the inhibition of development of rancid off-favours (Cornforth, 1996). Secondly, it reacts with myoglobin to give mononitrosylhaemochrome, which gives the characteristic pink color of cured meat. Thirdly, it inhibits the growth of food spoilage bacteria, and most importantly, *Clostridium botulinum* (Cammack *et al.*, 1999).

Nitrate and nitrite occur in the diet from numerous different sources (Knight *et al.*, 1987). Vegetables are major sources of nitrates, which is converted to nitrite when such foods are stored at room temperature (Weisburger *et al.*, 1975). Many plants, such as leafy vegetables or certain roots, accumulate extremely high concentrations of nitrate under favorable conditions of soil and water (Phillips, 1968; Heisler *et al.*, 1973). Since the 1970s there has been concern about a possible link between nitrite and cancer. There is no conclusive evidence that nitrite is directly carcinogenic (Cantor, 1997) but in high doses it has been implicated as a co-carcinogen (Schweinberg and Burkle, 1985).

Gastric cancer remains such a common neoplastic disease in many parts of the world (Kikuguwa and Nagao, 1990). Graham and co-workers (1990) found that high

ingestion of nitrate or nitrite in processed meats and fishes, heated fats and starch may be directly correlated with cancer risk. The correlation between nitrite ingestion and mortality from gastric cancer was reported (Fine *et al.*, 1976).

It is proposed that nitrate involves in the formation of carcinogenic *N*-nitroso compounds via two distinct phases of gastric carcinogenesis. Firstly, after ingestion and absorption in the stomach, nitrate is secreted in the saliva in concentrated form. Oral bacteria can then reduce nitrate to nitrite (Spiegelhalder *et al.*, 1976; Forman, 1989). In the second phase, nitrite is converted in the stomach to nitrous acid and reacts with certain substrates (amines, amides) to form carcinogenic *N*-nitroso compounds (Corres *et al.*, 1983) which frequently demonstrated to be carcinogenic in animals (Choi *et al.*, 1987; Forman, 1989).

2.2 Nitrite as A Converter for Direct Mutagen

Several investigators (Marqauard *et al.*, 1977; Llanes and Tannenbaum, 1982; Tomita *et al.*, 1982; Wakabayashi *et al.*, 1984) suggested that direct-acting mutagens/carcinogens formed from nitrite and the precursors of mutagen in the acid conditions of the stomach were possible candidates for the causation of human gastric cancer. For instance, fava beans (*Vicia faba* L.) commonly eaten in Columbia where gastric cancer incidence (Correa *et al.*, 1983) highly shown direct acting mutagenicity towards *Salmonella* strains after treated with nitrite in acid solutions (Llanes and Tannenbaum, 1982; Hoeven *et al.*, 1984)

Japan is the country that has the highest mortality rate of gastric cancer in the world (Marqauard *et al.*, 1977). Investigating on components of the Japanese diet, Wakabayashi and his associate (1983,1985) found that bean paste, fish sauce, pickled vegetables, sun-dried fishes and Chinese cabbage showed direct acting mutagenicity on *Salmonella typhimurium* TA100 after nitrite treatment. In addition, soy sauces, widely used as seasoning in Southeast Asia and Japan, were strongly mutagenic to *Salmonella typhimurium* TA100 after being interacted with 50 mM nitrite. Lin *et al.* (1979) found that soy sauce treated with nitrite in the range of 1000 to 10000 ppm was mutagenic in direct relation to nitrite concentration. However, soy beans themselves and soy sauce produced by acidic hydrolysis of soy bean showed no mutagenicity after treatment with nitrite.

Various food produced in Asia were reported on their direct acting mutagenicity after nitrite treatment. Kimchis, sun-dried fishes, sun-dried squid, soy sauces, fish sauces, bean paste 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. Salt pickled cabbage eaten by the Korean three times a day contained high level of *N*-nitroso compound after treatment with nitrite under simulated human stomach conditions. Thus, salted pickled cabbage may play an important role in the gastric cancer in Korea (Seel *et al.*, 1994). 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).

Kangsadalampai and Butryee (1995) found that nitrite treated products of natural thai food colors from 5 plants, namely *Clitorea ternatea* Linn., *Hibiscus sabdariffa* Linn., *Pandanus amaryllifolius* Roxb., Caramelized coconut sugar and *Carthamus tinctorius* Linn., and of a synthetic color, Ponceau 4R, exhibited their mutagenicity on *Salmonella typhimurium* both strains TA98 and TA100. Mutagenicity of the extracts of Thai medicinal plants after nitrite treatment was found on *Andrographis paniculata* Ness, *Carthamus tinctorius* Linn., *Cassia angustifolia* Vahl., *Cassia fistula* Linn., *Centella asiatica* Linn., *Curcuma domestica* Vahl., *Curcuma zedoaria* Rosc., *Cyperus rotumdus* Linn., *Oroxylum indicum* Vent. and *Zingiber officinale* Rosc. (Kangsadalampai *et al.*, 1995). A food additive, namely sorbic acid could react with nitrite to yield mutagens (Namiki *et al.*, 1981). Being treated with nitrite, pepper exhibited the strongest mutagenic activity in the Ames test, while nutmeg, chili pepper and laurel also showed strong activities (Namiki *et al.*, 1984). No mutagenicity was observed for spices alone. In addition, Shephard *et al.* (1993) reported that aspartame (artificial sweetener) nitrosated for 10-30 min with 40 mM nitrite (pH 3.5, 37°C) had mutagenic activity on *Salmonella typhimurium* strain TA100. Furthermore, the products formed by reacting with nitrite under gastric simulating condition (pH 3.0-3.5) of *Fomes japonica*, *Ganoderma applanatum*, *Acetobacter xylinum*

were mutagenic to both strain TA98 and TA100, while royal jelly was mutagenic to only strain TA98 (Katipagdeetham, 1996). 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.

2.3 Menstrual Reguratory and Haematinic Traditional Preparations

Many medicinal herbs in Thailand have been studied for decades, especially on the formulation as therapeutic remedies. Literature surveys (เทพนม เมืองแมน และคณะ, 2523.; เสงี่ยม พงษ์บุญรอด, 2493.; โสภิต ธรรมอารี และมณฑิรา ภัณฑ์เกยูร, 2525.) showed that the major components of most menstrual reguratory and haematinic traditional preparations are *Angelica sinensis* Diels (โกฐเชียง), *Ligusticum chuanxiong* Hort (โกฐหัวบัว), *Cinnamomum* sp. (อบเชย), , *Plumbago* sp. (เจตมูลเพลิง), *Cymbopogon citratus* Staf. (ตะไคร้)., *Lawsonia inermis* Linn. (เทียนขาว)., *Carthamus tinctorius* Linn. (คำฝอย). These plants have at least one of the following properties: 1.) induce abortion in both man and animals, being used as contraceptive, or regulated menstrual cycle. 2.) induce uterine contraction. 3.) Help uterine recovery after delivery. (Sroysa-ard, 1997).

Ungsurangsie *et al.* (1982) used *Bacillus subtilis* subtype H17 (rec^+) and M45 (rec^-) as mutation indices to reveal the mutagenicity of 31 herbal species. Some of the studied plant that were often found in menstrual regulatory and haematinic traditional preparations were *Cinnamomum* sp. (อบเชย), *Piper nigrum* Linn. (พริกไทย), *Piper longum*

Linn. (ดีปลี), *Myristica fragrans* Houtt. (ดอกจันทน์, ลูกจันทน์), *Cinnamomum zeylenicum* Nees. (อบเชยลังกา). They found that Ceylon cinnamon or *Cinnamomum zeylenicum* Nees. (อบเชยลังกา) was mutagenic in Ames test.

Mutagenicity of *Piper nigrum* Linn. (พริกไทย) and *Myristica fragrans* Houtt. (ดอกจันทน์, ลูกจันทน์). were discovered using Ames test by Namiki *et al.* (1984). These plants contained some indirect mutagens requiring hepatic mixed function oxidase to express its mutagenicity.

Piperine, the alkaloidal substance founded in pepper species, was revealed that it could induce infertility in mice because of abnormal function of uterine cells. (Piyachaturawat *et al.*, 1982).

Kangsadalampai and Rojanapo (แก้ว กังสดาลอำไพ และ วรรณี โรจณโพธิ์, 2531) studied the mutagenic potential of the menstrual regulatory and haematinic traditional preparations (8 recipes), using Ames test. The results showed that in the *in vitro* Ames test only the drugs interacted with nitrite, either in acid condition or gastric secretion, demonstrated the mutagenic potential risk to the consumer.

Chaiyakum *et al* (อาภรณี ไชยาคำ และคณะ, 2526) found that Thai pregnant experienced in using the menstrual regulatory and haematinic traditional preparations had double risk in having congenital birth defect babies. Congenital abnormality is one of the important factors of fetal death rate.

2.4 Antimutagenic Activity of Herbs

Herbs have been used as food and for medicinal purposes for centuries. Research interest has focused on various herbs that may be useful adjuncts in helping reduce the risk of cardiovascular and cancer. A wide variety of active phytochemicals, including the flavonoids, terpenoids, lignans, sulfides, polyphenolics, carotenoids, coumarins, saponins, plant sterols, curcumins, and phthalides have been identified (Craig, 1999). In addition, some antimutagenicity compounds, such as chlorophylls, chlorophyllin and haemin, required complex formation with mutagens to express for their activity (Duh and Yen, 1997).

Roselle (*Hibiscus sabdariffa* Linn.), a member of the Malvaceae family, is a tropical plant of considerable economic potential. Utilization of roselle extract may include natural food colorants, emulsions for carbonated drinks (Francis, 1975). Thai people used for making cold drinks that have been used effectively in folk medicines against hypertention, pyrexia and liver disorders. The flowers contains gossipetin, anthocyanin, and glucoside hibicin, which may have diuretic and choleretic effects, decreasing the viscosity of the blood, reducing blood pressure and stimulating intestinal peristalsis. The drink made by boiling the calyx in water, is said to be a folk remedy for cancer.

Dried-flowers minus-ovary of roselle (Karkade sorrel) contains 13% of a mixture of citric, malic acid, anthocyanins, and gossipetin (hydroxyflavone), 15.3% hisbiscic acid ($C_6H_6O_7$) (Nakamura and Yamamoto, 1982), hibiscin, and phytosterols and 0.004-

0.005% ascorbic acid. Petals yield the flavonal glucoside hibiscritin, which yields a crystalline aglyconehibiscetin ($C_{15}H_{10}O_9$). The calyx of roselle possesses high contents of phenolic compounds and exhibits reducing power. The pigments contained in the flowers *Hibiscus* species are anthocyanins (phenolic natural pigments) such as cyanidin-3-glucoside and delphinidin-3-glucoside (Du and Francis, 1973) which may act as antioxidant or via other mechanisms contributing to anticarcinogenicity (Ho, 1992). The roselle extract may play an important role in protecting against damage to cell membrane function, as a result of lowering the level of lipid peroxide (Osawa *et al.*, 1985). The water extract of roselle also showed good hydrogen donating abilities, indicating that it had free radical scavenging activity. No mutagenicity of the water extracts of roselle was found in *Salmonella typhimurium* strains TA98 and TA100, either with or without metabolic activation (Duh and Yen, 1997). The 80% ethanol extract of roselle reduced about 60-90% of the mutagenicity induced by 2-amino-1 methyl-6-phenylimidazo (PhIP) and other IQ in *Salmonella* mutation assay (Chewonarin *et al.*, 1999). On the other hand, Kangsadalampai and Butryee (1995) found that roselle exhibited mutagenicity to *Salmonella typhimurium* both strains TA98 and TA100 after nitrite treatment.

Chrysanthemum flower (*Chrysanthemum morifolium* Hemsl.) is a member of the Compositae family. Chrysanthemum tea is a very popular cold beverage in Asia and is usually sold in paper carton. Because of its cooling properties, it is especially popular during the summer. The traditional therapeutic use of this herb is to relieve certain

hypertensive symptom such as headache, insomnia and dizziness. They open up the blood vessels and allow blood to flow more freely throughout the body (Duke, 1992). It is also widely used in the treatment of common cold, influenza and meningitis. The chemical constituents consist of three sesquiterpene guaianolides, angeloylcumambrin B, arteglasin A and angeloylajadin. Duh (1999) found that the water extract of the Chrysanthemum flower could be recommended as a potential source of natural antioxidants.

Safflower, False saffron (*Carthamus tinctorius* Linn.), a member of the Compositae family, is a widely used traditional Chinese plant medicine. It has the function of promoting blood circulation (Yin and He, 2000). Flowers considered diaphoretic, emmenagogue, laxative, sedative, stimulant, in large doses laxative; used as a substitute or adulterant for saffron in treating measles, scarlatina, and other exanthematous diseases. In China, it is prescribed as uterine astringent in dysmenorrhea (Keys, 1976). In Iran, the oil used as a treated product of safflower exhibited mutagenicity to both strains of *Salmonella typhimurium* (TA98 and TA100).

Bael fruit (*Aegle marmelos* (Linn.) Corr.) is a member of the Rutaceae family. The boiling water extract of dried slices of young fruit is used for its antidiarrheal effect that might be contributed by pectin, mucous substance, tannin and bitter ingredients. The chief constituents appear to be mucilage and pectin that contain in the pulp of unripe fruit; the ripe fruit differs in yielding a tannin reaction and possessing a distinct aroma. Antiprotozoal activity has also been shown (Chopra, 1982)

Asiatic pennywort (*Centella asiatica* (Linn.) Urban), a member of the Umbelliferae family, is a medicinal plant that has been in use since prehistoric times. There are a number of reports on the chemical components in *Centella asiatica* (Linn.)Urban (Basu and Rastogi, 1967; Singh and Rastogi, 1968; Rao and Seshadri, 1969). The group of compounds commonly found is a bitter principle, vallerin and a mixture of triterpenoid glycosides, namely madecassoside and asiaticoside. It also contains traces of alkaloid, sitosterol, tannin, resin, volatile oil and pectin (พเยาว์ เหมือนวงษ์ญาติ, 2529; วันดี กฤษณพันธ์, 2537; D'Amelio, 1987; Reynolds, 1989; Brinkhaus *et al.*, 2000). *Centella asiatica* has long been used for many thousand years as tonic juice, diuretic, antipyretic, and also treatment of sore throat and thirst (เสงี่ยม พงษ์บุญรอด, 2493; วันดี กฤษณพันธ์, 2537). It has also traditionally been used externally as a poultice for burns, inflammations, wounds and ulcer (วันดี กฤษณพันธ์, 2537; Brinkhaus *et al.*, 2000).

White mulberry (*Morus alba* Linn.) is a member of the Moraceae family. Trees are extensively grown (e.g. southern Europe, India) for their leaves as food for silkworms. Fruits may be eaten raw or cooked. Fruits are an ingredient of a particularly seductive drink known as mulberry wine. Mulberry leaves are sometimes eaten as a vegetable and are useful as a cattle fodder (Reed, 1976). Medicinally, fruits are laxative, refrigerant in fevers, and used locally as remedy for sore throat, dyspepsia, and melancholia. Roots and bark are purgative, anthelmintic, and astringent; leaves considered disphoretic and emollient; a decoction of leaves being used as a gargle for inflammation of throat (Reed, 1976).

2.5 The *Salmonella* Mutagenic Assay (Ames test)

Bacterial mutagenicity assays, especially the Ames test (*Salmonella typhimurium* his⁻ reversion assay), have been used worldwide, in research laboratories. Their application is motivated by several goals; the identification of genotoxic hazards; the quantitation and regulation of health risks resulting from environmental chemical exposures; and the elucidation of the biochemical mechanisms of mutagenesis. The potential of this method for use as a bioassay for the development of safe, useful chemicals raised many questions about the extent to which this kind of approach should be used in a program aimed at cancer prevention.

The *Salmonella* histidine reverse assay is based on the used of several selected histidine dependence (auxotrophy) to histidine independence (prototrophy) at an increased frequency in the presence of a mutagen. The test detects a wide variety of mutagens, including many that require an exogenous metabolic activation system. The test is used as a screen for mutagenic activity of pure compounds, complex mixtures, and body fluids. At present the most commonly used *Salmonella* strains are TA1535, TA1537, TA1538, TA98 and TA100. The number and type of strains used depend upon the availability and type of samples, the focus of the study, and previous knowledge concerning the test material. In addition to having a mutation in one of the genes of the histidine operon, tester strains frequently have mutations that impart other specific characteristics to the tester strain. One mutation (*rfa*) lead to a defective lipopolysaccharide coat; another is a deletion of genes involved in the synthesis of the

vitamin biotin (*bio*) and in the excision repair of DNA damage (*uvr* B). The *rfa* mutation increases the permeability of the strains to large molecules, thereby increasing the mutagenicity and/or toxic effects of these chemicals. The *uvr* B mutation leads to a reduced level of error-free repair of some types of DNA damage and thereby enhances the strain sensitivity to certain chemical and physical mutagens. Strain TA100 is derived from TA1535 by the introduction of the plasmid pKM101, which increase the sensitivity of mutagen detection by enhancing error-prone DNA repair. The presence of this plasmid makes TA100 respond to some frameshift mutagens as well as base-pair substitution mutagens. Strain TA98 is derived from TA1538 by introduction of plasmid pKM101. All tester strains should be maintained and stored according to published method (Ames *et al.*, 1975; Maron and Ames, 1983). They should be analyzed on a frequent and rational basis for each characteristic that could affect the test. For example, strain identification could include the following: histidine and biotin requirement, UV sensitivity (presence of the *uvr* B deletion), crystal violet sensitivity (presence of the *rfa* mutation), ampicillin and/or tetracycline resistance (presence of the apprppriate plasmid), spontaneous reversion frequency, and reversion characteristics to various positive controls.

Three of the most important his alleles found in the Ames tester strains (Hartman *et al.*, 1986) are listed below, along with typical strains bearing the allele; the nature of the mutation in the target gene; and the most common pathway for its reversion:

hisD3052; TA1538, TA98: -1 frameshift; ΔGpC frameshift in $(GC)_4$ run

hisG46; TA1535, TA100: missense; base-substitution at G:C base-pair

hisG428; TA102, TA104, TA2659: ochre; base-substitution at A:T base-pair

Each Ames test strain evaluates mutagenic activity at a specific (reversion) target sequence. In the case of the frameshift allele *hisD3052* revertants bearing many different sequence change (spanning a region of more than 50 bp) can be recovered: of course each such event restores the correct reading frame. Multiple classes of revertants of the base-substitution alleles can also recovered, including transition, transversion, and some extragenic suppressor mutations

McCann and Ames (1977) discussed several aspects of the experimental basis for their current assessment of the value of the test as a useful predictive tool:

1. The predictive value of the test as an indicator of carcinogenic potential, including both the strengths and weaknesses of the test at this stage in its development.

2. Current applications of the test method to problems that were not approachable using conventional animal test methods.

3. Some of the environmental chemicals that have already been pinpointed as potential carcinogens by the test and the current status of carcinogenicity tests of these chemicals in animals.

4. The evidence that the correlation between carcinogenicity and mutagenicity in the Salmonella test reflected more than a useful concidence and fitted into a compelling collection of evidence supporting a central role for somatic mutation in the initiation of human cancer.

2.6 The Mutagenicity Test (Preincubation Method) Using *Salmonella typhimurium*

Some mutagen, such as dimethylnitrosamine and diethylnitrosamine are poorly detected in the standard plate incorporation assay and should be tested using a modification of the standard procedure. The most widely used test modification is the preincubation assay first described by Yahagi *et al.* (1975), in which carcinogenic azo dyes were found to be mutagenic. They incubated the mutagen and bacteria for 20-30 min at 37°C and then added the top agar. The assay has been also used to detect the mutagenicity of 10 carcinogenic nitrosamines (Yahagi *et al.*, 1977) and several carcinogenic alkaloids (Yamanaka *et al.*, 1979). The mutagenic activity of aflatoxin B1, benzidine, benzo[a]pyrene and methylmethane sulfonate has been determined using both plate incorporation and preincubation procedures and in all cases the preincubation assay is of equal or greater sensitivity than the plate incorporation assay (Matsushima *et al.*, 1980). The increased activity is attributed to the fact that the test compound and bacteria are incubated at higher concentration in the preincubation assay than in the standard plate incorporation test (Prival, 1979). The procedure described below is based on recommendation of Matsushima *et al.* (1980).

The preincubation modification can be used routinely or when inconclusive results are obtained in the standard plate incorporation assay. This assay requires an extra step and therefore involves more work than the standard test. Nevertheless, many laboratories use it routinely because of the increased sensitivity towards some

compounds. Its use in screening assays has been recommended by De Serres and Shelby (1979).