CHAPTER 1

INTRODUCTION

1.1 Statement of the problems

Some studies of antimutagens in some Thai medicinal plants have already been performed. It is anticipated that Thai medicinal plants may be a useful source for cancer chemoprevention. Using a short term screening test, named Salmonella mutation test, possible antimutagens were first found in lemon grass (Cymbopogon citratus), Kuffir lime (Citrus hystrix), and greater galangal (Alpinia galanga) (Vinitketkumnuen, et al, 1993). Recently, antimutagenic extracts from lemon grass were examined in anti-DMN induced DNA strand breakage in rats, using the alkaline elution method. Interestingly, it was found that antimutagenic extracts could inhibit DMN-induced DNA strand breakage (Vinitketkumnuen, et al, 1991).

Cancer and certain genetic mutations are often due to factors regarding life-style, especially the intake of mutagens, carcinogens and cancer promoters, some of which are dietary in origin (Ames, 1983; Hayatsu, et al, 1988; Miller and Miller, 1986; Peter, 1982; Sugimura, 1982). Furthermore, there is growing evidence that these conditions may be due, in part, to less than optimal intake of dietary antimutagens, anticarcinogens or anti-tumor promoters (Higginson, 1983; Higginson, 1988; Hinnekens, 1980; Ramel, 1986; Wattenberg, 1985; Wynder, 1977). Evidence has accumulated to suggest the presence of antimutagens in Thai foods (Meevatee, et al, 1993; Pinseang, 1993; Rojanapo, et al, 1990 ; Rojanapo and Tepsuwan, 1992; Vinitketkumnuen, et al, 1992). In Thailand, certain plants have

long been used as a source of medicine as well as dietary components. Scientists from various countries have obtained new drugs from Thai plants, and these drugs have been useful in treating many diseases.

Present investigations aim to obtain an extract of lemon grass (Cymbopogon citratus) by 80% ethanol extraction; this extract will be examined to look for antimutagens, in vitro cytotoxic effects and in vivo antitumor activity against cancer cells. Previous studies indicated that the optimal antimutagenic activity was in a methanolic extract of lemon grass (Lomsri, et al, 1991). However, in this study the ethanolic extract was used in order to prevent toxic effects to animal ; due to methanol.

Lemon grass was chosen for this study because of the following criteria :

1. It is commonly available in Thailand (and possibly in other regions of the world).
2. It is widely used, in part or in whole, as a dietary or medicinal component.
3. It has been shown by previous studies to possess some biological activity which suggest antimutagenicity.

Previous studies have found that methanolic extract of lemon grass has decreased chromosomal aberration in human lymphocytes induced by mitomycin C (Meevatee, et al, 1993), inhibited micronucleus formation in rat bone marrow induced with methylethane sulfonate, dimethylnitrosamine, mitomycin C and cyclophosphamide (Lim, et al, 1988; Pinseang, 1993). This extract inhibited DNA strand breakage in livers of rats which were given dimethylnitrosamine (Vinitketkumnuen, et al,1991). Pharmacological studies also indicated that lemon grass tea infusion given to rats for 2 months did not cause any toxicity (Carlini,

et al, 1986; Souza, et al, 1986). The level of glucose, urea, creatine, cholesterol, triglycerides, lipids, total bilirubin, indirect bilirubin, GOT, GPT, alkaline phosphatase, total protein, albumin, LDH and CPK in the plasma of humans who had received lemon grass tea infusion did not differ from the normal level (Leite, et al, 1986). The decoction of lemon grass leaves showed dose-related hypotensive effects when given orally to rats (Cabajal, et al, 1989). In addition, essential oils in lemon grass induced GST, a detoxificating enzyme, activity in mice, small intestinal mucosa (Luke and Boling, 1991). The methanolic extract also inhibited the activity of Epstein-Barr virus (EBV) induced by tumor promoter, teleocidin (Murakami, et al, 1993a ; Murakami, et al, 1993b). Subsequently, an effective anti-tumor promoter was identified as geranial. Many therapeutically effective substances in lemon grass were isolated and identified, such as geranial (α-citral) and neral (β-citral). These substances were shown to have an antibacterial action against gram-negative and gram-positive organisms (Onawuni, et al, 1984). Another substance, myrcene, had an analgesic activity against carrangenin and prostaglandin E_2 (Lorenzetti, et al, 1991).

With increasing evidences that diet may play an influential role in cancer development, it seemed appropriate to establish ways to categorize foods according to their potential for tumor promotion or tumor inhibition. The present study is an effort to determine anticarcinogenicity of putative antimutagenic lemon grass. Further work will be directed toward isolation and identification of dietary consituents which have anti-tumor forming properties.

It is hoped that this study would provide useful data in the on-going search for information on the dietary influence on the incidence of cancer incidence. More

experiment data are needed to provide stronger validity for dietary guidance for those who are seeking advice in developing more healthful eating habits which would lessen the long-term risk for cancer.

1.2. Literature reviews

1.2.1. Environmental mutagens

1.2.1.1. Chemicals that require activation by P450 cytochrome dependent monooxygenase

1. AFB_1 [Aflatoxin B_1]

Aflatoxins are primarily metabolized by the microsomal mixed function oxygenase system. These enzymes catalyze the oxidative metabolism of AFB_1, resulting in the formation of various hydroxylated derivatives as well as unstable, highly reactive epoxide metabolite, the ultimate carcinogen. Once produced, aflatoxin-8,9-oxide reacts with N^7atom of guanine in DNA and RNA to form 8,9-dihydro 8(N^7-guanyl)9-hydroxy AFB_1 as well as lysine residues in protein such as albumin. Also AFB_1 can conjugates with glutathione to form AFB_1-GSH conjugate, which is excreted (Figure 2). Epidemiologic studies have been published on the association of aflatoxin exposure and liver cancer (Lin, et al, 1978; Neal and Colley, 1978; Tadi, et al, 1991; Water, et al, 1990) .

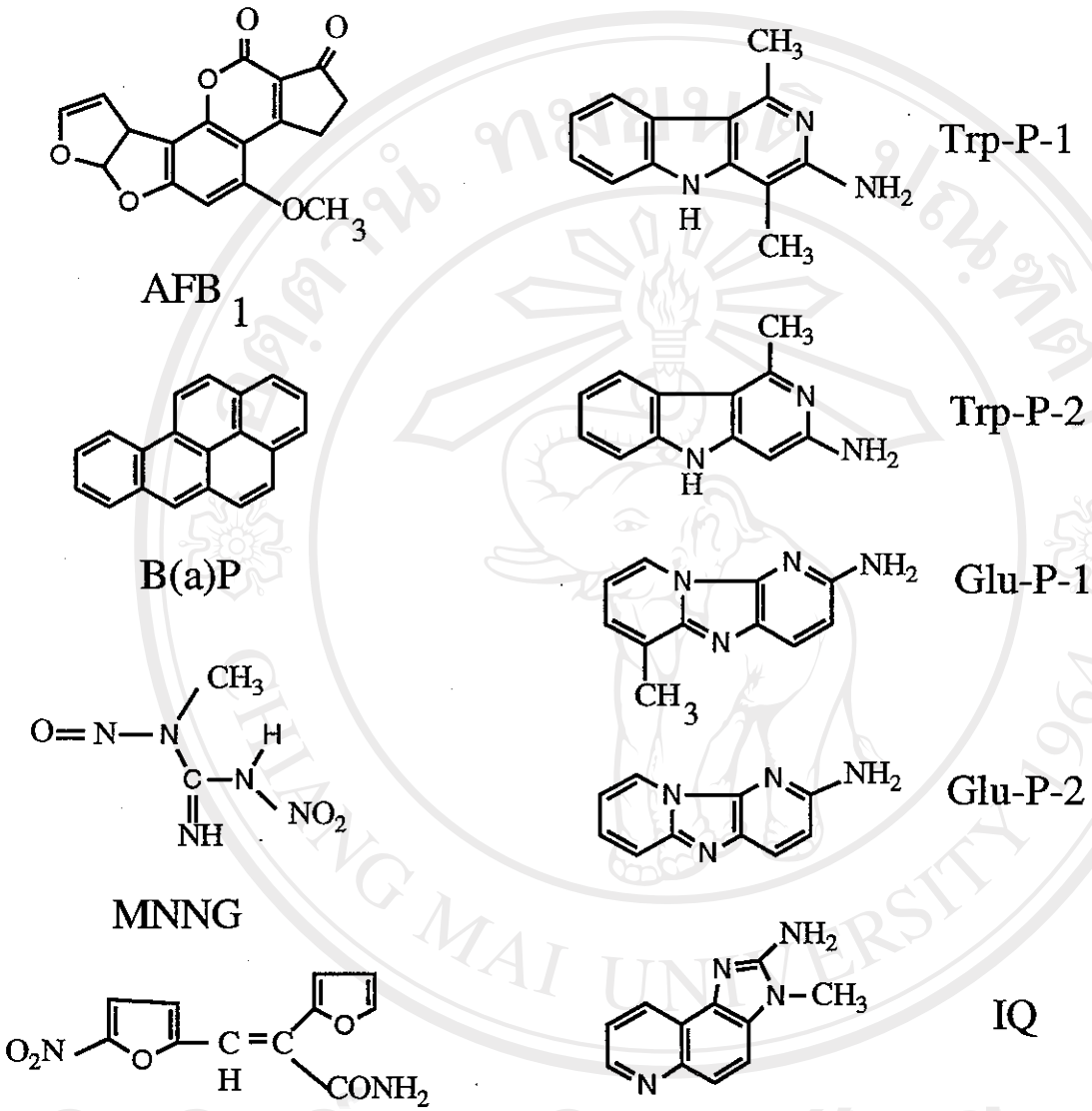

Figure 1 Structure of some environmental mutagens.

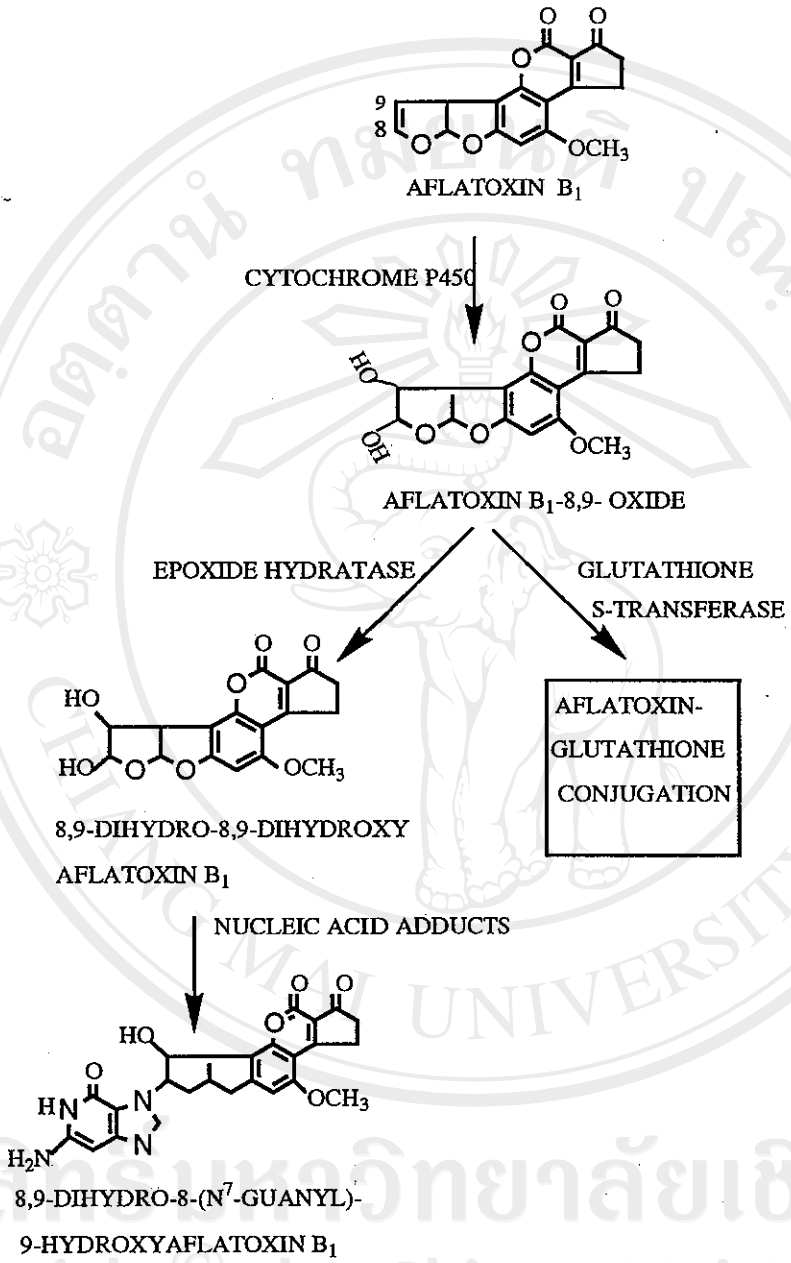

Figure 2 Metabolic activation of aflatoxin B_1

2. Heterocyclic aromatic amines

The normal cooking and processing of meat-containing food generates a number of chemicals which are positive in bacterial mutagenicity assay (Sugimura, et al, 1983). Among these are several heterocyclic amines of imidazoquinoline (IQ) and methylimidazoquinoxaline (MeIQx) derivatives (Sugimura, 1992). These compounds are produced by heating protein food of animal origin and are detectable after ordinary cooking. The precursors of IQ and MeIQx compounds are creatine, which is abundant in fish and meat, amino acids and sugar (Kleman, et al, 1989). In addition, pyrolysis of tryptophan and glutamic acid generates pyridoindole (Trp-P-1 and Trp-P-2) and dipyridoimidazole (Glu-P-1 and Glu-P-2) derivatives, respectively (Ames, et al, 1975; Sugimura, 1986). Amino groups of both IQ and non-IQ type heterocyclic amines are metabolically activated to N-hydroxyamino groups by cytochrome P450 IA2 (Gonzalez, 1989; Kato, et al,1987). The N-hydroxy amino derivatives are further modified by esterification with acetic acid, sulfuric acid, and proline, and these forms produce DNA adducts (Kato, et al, 1987; McManus, et al, 1988). The ultimate reactive forms of Trp-P-2, Glu-P-1, IQ and PhI from adducts at the C-8 position of guanine to form 8-hydroxyguanosine in place of deoxyguanosine residue (Snyderwine, et al, 1988). This base specificity is consistent with the finding that in heterocyclic amine-induced tumors, mutations in the *ras* and p53 genes occur most often at G:C base pairs (Makino, et al, 1992a; Makino, et al, 1992b) (Figure 3).

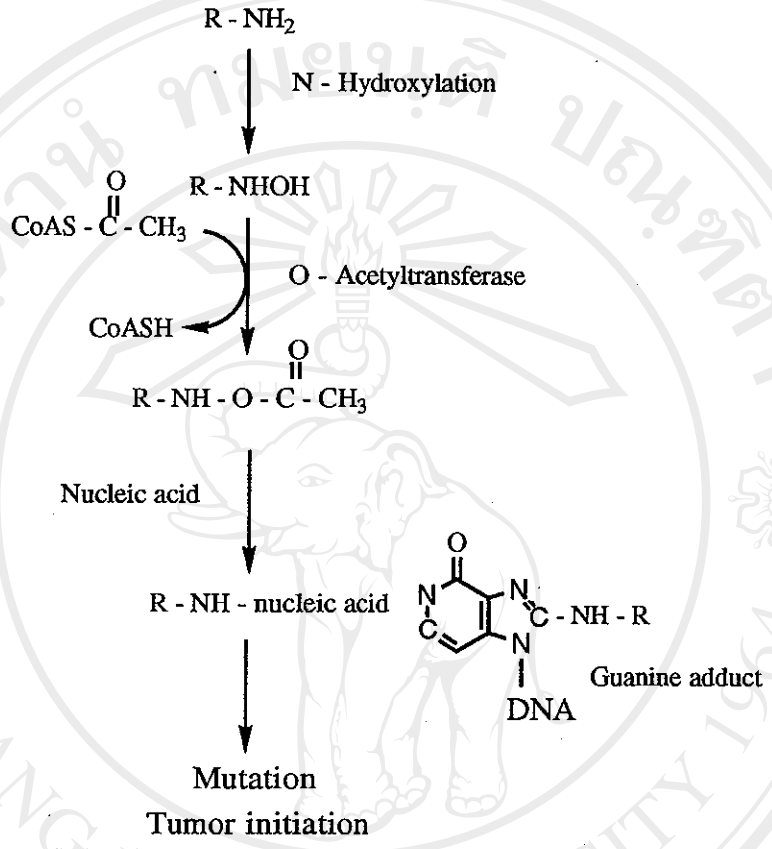

Figure 3 Metabolic activation of heterocyclic aromatic amines.

3. B(a)P [Benzo(a)pyrene]

The metabolism of B(a)P, a polycyclic aromatic hydrocarbon, results in the formation of reactive epoxides at various positions on the hydrocarbon skeleton by cytochrome P450-dependent mix function oxidase system (Fiala, et al, 1985). These epoxides isomerize spontaneously to phenol or are hydrated via epoxide hydratase to dihydrodiol. Detoxification occurs through conjugation of the epoxide with GSH or of the phenols and dihydrodiol with biologically active forms of sulfuric acid and glucuronic acids. The metabolic activation of B(a)P to its principle carcinogenic intermediate appear to be initiated by specific epoxidation at C7 and 8 of B(a)P. 7, 8-Dihydrodiol is a substrate for further epoxidation at the 9,10-position in a sterospecific manner. Although several cytochrome P450 isozymes are involved, B(a)P is critically metabolized at the 7, 8 position by P448 or P450IA1, the isozyme induced by 3-methylcholanthrene (Gozukara, et al, 1982). The very high electrophilic reactivity of the 7,8 diol-9, 10-epoxide is the ultimate mutagen /carcinogen (Buening, et al, 1978) (Figure 4).

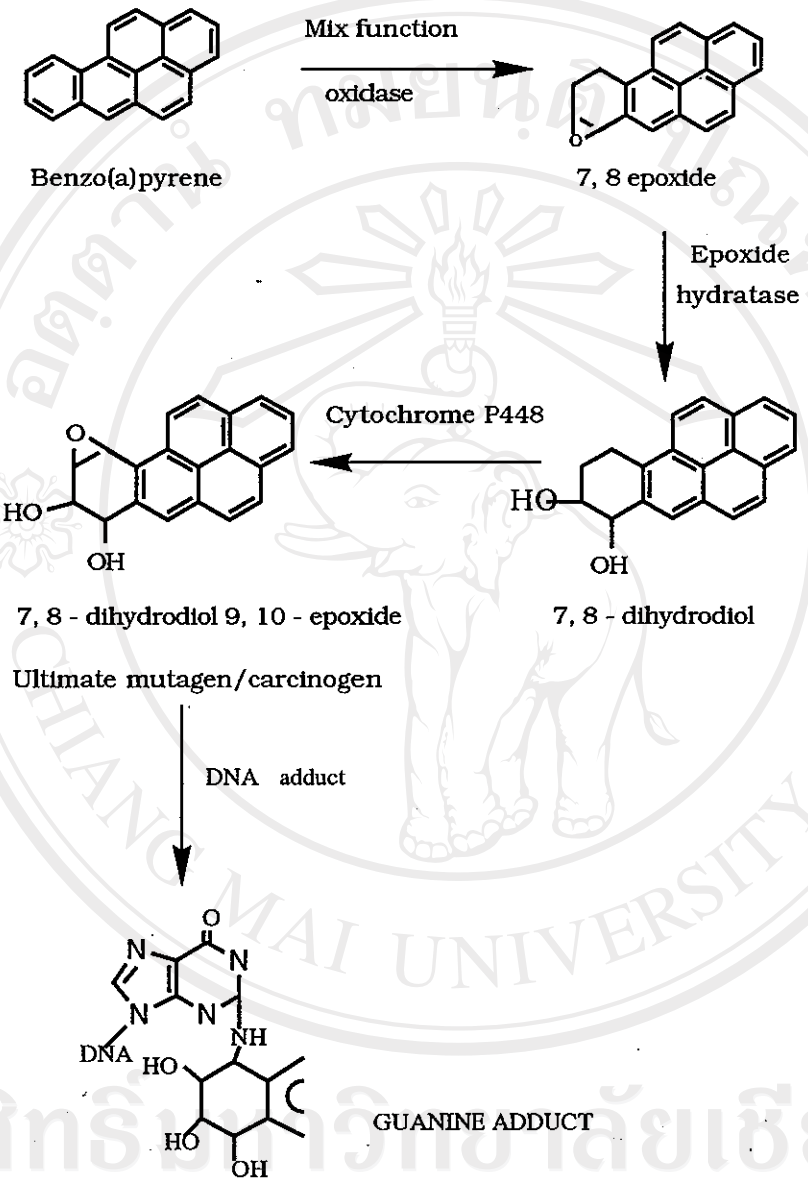

Figure 4 Metabolic activation of benzo(a)pyrene

1.2.1.2. Direct acting mutagens

1. MNNG [N- methyl-N'-nitro-N-nitrosoguanidine]

MNNG, a direct acting alkylating agent, does not require enzymatic activation, since nonenzymatic reaction with water or other cellular nucleophiles under physiologic conditions results in the formation of alkyl-diazonium ion (Magee, et al, 1976). Mutagenesis induced by MNNG is largely dependent on the ability to methylate DNA bases at the oxygen atom. A major mutagenic and carcinogenic lesion is methylated at oxygen position 6 of guanine base by unimolecular nucleophilic substitution mechanism (Sn 1) (Figure 5). O^6-methylguanine can pair with thymine instead of cytosine during DNA replication, resulting in G:C to A:T transition mutation (Matic, et al, 1991).

2. AF-2 [2-(2-furyl)-3-(5-nitro-2-furyl)furamide]

AF-2, a nitrofuran derivative, was once used as a food preservative in Japan. It was a direct acting mutagen, inducing mutation in the absence of metabolic activation systems (Sugimura, 1986). However, there is good evidence that metabolic activation by the endogenous system is a prerequisite to induction of DNA damage and mutation. Activation of this compound is a reductive process catalyzed by flavoprotein enzymes which transfer electrons from NADPH or NADH to the nitro group (Figure 6). These enzymes which have nitro-reductase activity are widely distributed and occur in *E.coli*, *S.typhimurium*, and in many mammalian tissus and culture cells (McCalla, 1981). Reductive activation of AF-2 takes place at a much more rapid pace in bacteria than in mammalian cells and tissues.

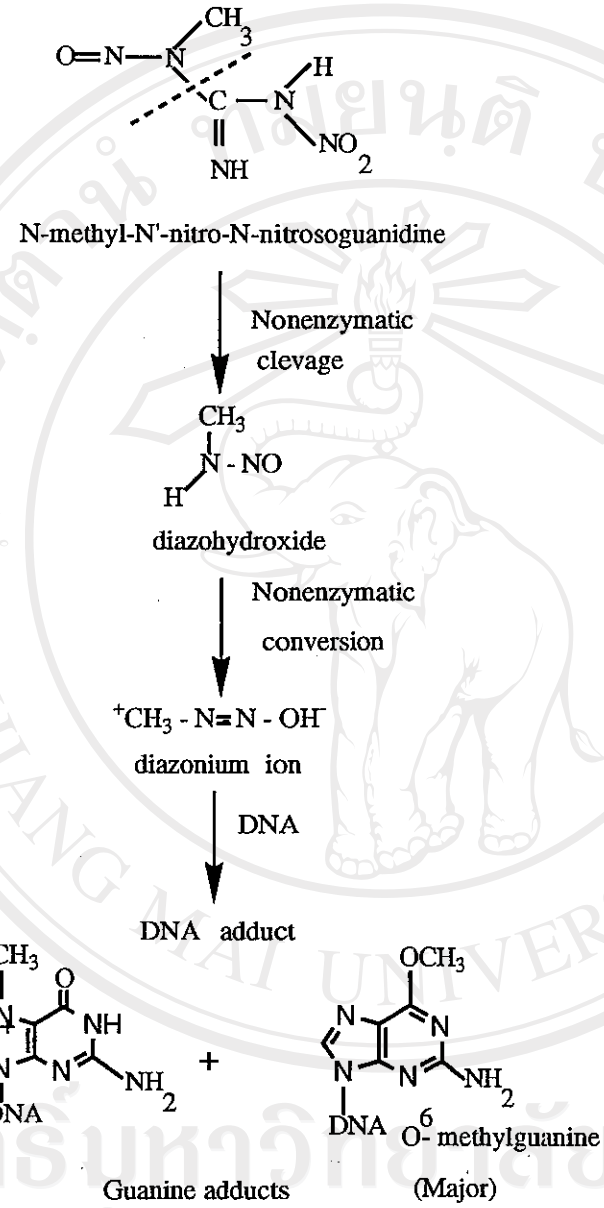

Figure 5 Metabolic activation of N-methyl-N'-nitro-N-nitrosoguanidine

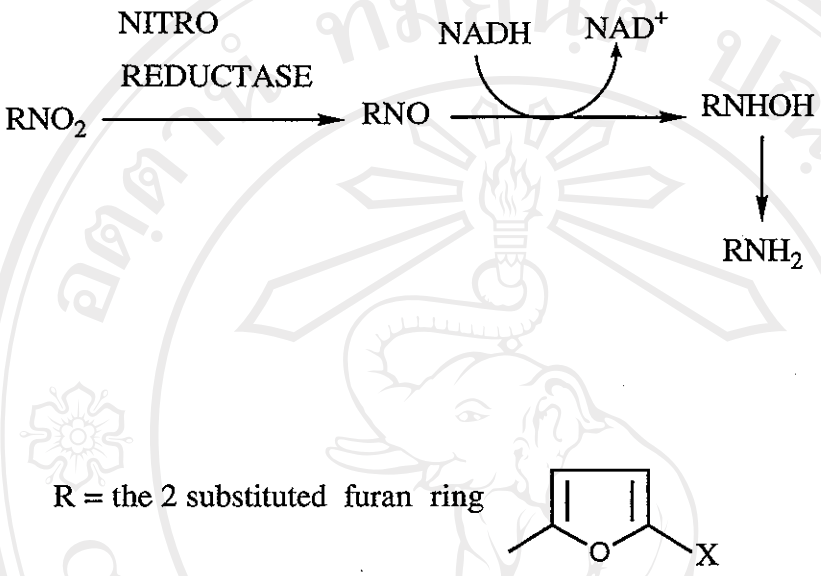

Figure 6 Metabolic activation of 2-(2-furyl)-3-(5-nitro-2-furyl)-furamide.

1.2.2. Bacterial mutagenesis and its role in the identification of potential animal carcinogens.

The increasing number of chemicals, their spreads into the human environment, and their consumption by human lead to quantitative evaluations of their potential adverse effects. Short term tests have become predominantly biological assay for detecting and assessing genotoxic and mutagenic effects. Their goals are to determine genetic alteration caused by reactive chemical compounds (Pool, 1988) and to contribute directly to the quantification of human risk (Edler, 1992). Most chemical carcinogens are mutagens (Lawley, 1989). This has been demonstrated using the Ames assay and other tests. At a molecular level, transitions, transversion, and other types of mutation have been shown to occur following exposure of certain bacteria to ultimate carcinogens. It has been assumed that some types of cancer are due to mutations in somatic cells that affect key regulatory processes.

Since testing the carcinogenicity of chemical in animals is slow and expensive, assay for screening the potential carcinogenicity of chemical compounds has been developed. Many tests are based on detection of the mutagenicity of chemical carcinogens. Such assays are more rapid and less expensive than detecting tumors in animals. None is ideal, since the ultimate test of a carcinogen is to show that it causes tumor in animal. However, one assay base on detecting mutagenicity, the Ames assay, has proved useful in screening for potential carcinogens. This assay uses a specially constructed strain of *Salmonella typhimurium* that has a mutation (His^-) in a gene that codes for one of the enzymes involved in the synthesis of histidine. Thus, these particular salmonellae cannot

synthesize histidine, which must be present in the medium for growth to occur. When a mutation caused by a carcinogen occurs at the site of the His^- mutation, the latter mutation can restore its reading sequence and convert it to His^+ mutation. The progeny from bacteria containing such a reverse mutation can now synthesize histidine and thus grow in a medium lacking it. Such salmonellae can be detected as readily observable and quantifiable colonies growing on agar plates.

One problem ith the use of bacteria in mutagenicity tests is that they do not contain the spectrum of monooxygenases found in higher animals. Thus, if a compound requires activation to become a mutagenic or carcinogenic species, this may not occur when bacteria are used. Ames circumvented this problem by incubating the tested agents in postmitochondrial supernantant of rat liver. The S9 fraction, which is the supernatant fraction after centrifuging a rat liver homogenate at 9000g for a suitable period of time, contains most of the various monoxygenases and other enzymes required to activate potential mutagens and carcinogens. The Ames assay identified approximately 90% of known carcinogens (Maron and Ames, 1983).

1.2.3. Dietary inhibitors of carcinogenesis

Continuous DNA damage is expected to progressively alter the genomic sequence, as indicated by the effect of aging, to the extent that it contributes to the development of cancer, such as by activation of oncogenes and deactivation of suppressor genes (Simic and Bergtold, 1991). The studies of neoplastic transformation (Land, et al, 1983; Newbold and Overell, 1983) suggest that at least two mutagenic events are involved in the transformation of cells leading to

the initiation of cancer. Therefore, gene and chromosomal mutations are important factors in carcinogenesis (Ames, 1979; Ramel, 1986). The incidence of cancer can be reduced by decreasing the rate of mutation. In the last decade, great interest has developed in substances which posses properties that directly or indirectly reduce or eliminate the mutagenic activity of other chemicals. These substances are called "antimutagens" and many of these inhibitors occur naturally in food or elsewhere in nature (Wattenberg, 1983). Antimutagens are classified into 2 categories, "desmutagens" and "bio-antimutagens" according to their mode of action (Kada, et al, 1986; Kada and Shimoi, 1986). The former inactivates mutagens by chemical or enzymatical interaction. The latter suppresses the process of mutagenesis after DNA is damaged by mutagens. Therefore, it has been suggested that inhibitors of mutagenesis are related to anticarcinogenic effects which have a role for several reasons.

Inhibitors for mutagenesis have often been found by using Salmonella mutation assay. Many papers have reported that many substances exhibit inhibitory effects on mutagenesis. For instance, chlorophyll and chlorophyll- containing vegetables exhibit antimytagenic activity (Lin, et al, 1978) as well as green tea (Thes sinensis) containing epigallocatechin gallate (Kada, 1981). Vanillin in vanilla showes a strong bio-antimutagenic effect on mutagenesis induced by 4-NQO , AF-2 and capten (Kuroda and Inoue, 1988). Cinnamaldehyde in cinnamon shows a marked antimutagenic effect against mutations induced by UV and UV-mimetic mutagens such as 4-NQO and AF-2 (Ohta, et al, 1983). Furthermore, many papers have reported that many substances show anticarcinogenic effects in animal model. These include phenethyl isothiocyanate in many cruciferous vegetables

which has been shown to inhibit carcinogenesis when administered before or during exposure to nitrosamine and polycyclic aromatic hydrocarbons (Guo, et al, 1992).

1.2.4. Rationale and strategies for chemoprevention of human cancer

It is well known that various natural and man-made mutagens and carcinogens exist in the environment (Ames, 1979), even in our ordinary foodstuff (Ames, 1983 ;Peter, 1982; Ramel, et al, 1986). The potential health impact of dietary and other environmental mutagens includes genetic disorders, birth defects, cancer and heart disease (Ames, 1979). There are a considerable number of suspect mutagens/carcinogens in the environment that have been identified by virtue of mutagenicity testing.

Health protection against genotoxic hazards from chemicals in the environment can be accomplished in many ways. The potential chemoprevention as a means of halting or delaying the process of carcinogenesis is assessed as a strategy for reducing the incidence of human cancer. The concept of the chemoprevention of cancer as originally proposed referred to the prevention of cancer by the use of pharmacological agents to inhibit or reverse the process of carcinogenesis (Sporn and Newton, 1979). However, a number of different types of prevention studies can be considered (Bertram, et al, 1987), these are divided into 3 steps. First, primary prevention is the prevention of exposure before it occurs. Examples would include "don't smoke", "use sunscreen" ,"decrease fat in your diet", which are generally prescriptive in nature and do not involve active chemoprevention. Furthermore, this strategy has been possible with occupational exposure to compounds such as vinyl chloride monomer and 2-napthylamine.

However, in view of the widespread occurence of carcinogenic or mutagenic compounds in food as well as in other parts of the environment, effective elimination of exposure to many genotoxic chemicals is not feasible. Thus, secondary intervention is also important. Thus involves the use of an agent to prevent neoplasia resulting from carcinogen exposure. Ordinary examples would include diets which have antimutagens/ anticarcinogens such as fruits and vegetables containing vitamin A and C, in the high risk cancer groups such as smokers, asbestos workers or aniline dye workers. Tertiary intervention corresponds to use of an agent in individuals with already established neoplasia, who do not yet have cancer. These would to include individuals with such conditions as dysplasia of the bladder, cervix, esophagous or lung or adenomatous colonic polyps. There is a narrow distinction between intervention in preneoplasia and adjuvent therapy of early cancer with antiproliferative agents such as antiestrogen in breast cancer. On mechanistic grounds, they (Ramel,et al, 1986) have exclude this latter agent from consideration. Intervention by use of pure compounds has the adventage of specific choice; however, it requires knowledge of the compounds administered and control of dosage to prevent side effects.

1.3. Purpose of the study

The aims of this thesis were

1. To investigate antimutagenic activity of lemon grass extract in Salmonella mutation assay.
2. To determine cytotoxic effects of lemon grass on some cancer cell lines.
3. To study antitumor activity of lemon grass in mice transplanted with fibrosarcoma.
4. To partially purify active antimutagenic substances from lemon grass.