Chapter 1
Introduction

1.1 Statement of problem

Colorectal cancer is one of main cause of death from cancer in Western countries, and this may also soon be the case in Asian countries due to the rapid influence of Western lifestyles. The progressive introduction of Western dietary habits, especially an increasing fat intake and decreasing carbohydrate intake, has been paralleled by an increasing in colon and breast cancer (Tajima *et al.*, 1985). Epidemiologic studies indicated a statistically significant inverse association between cancer development and consumption of fruits and/or vegetables (Block *et al.*, 1992). Diets in general contain antimutagenic or anticarcinogenic compounds as well as mutagenic or carcinogenic agents. These antimutagens and anticarcinogens may inhibit one or more stages of carcinogenesis process and delay or prevent cancer development.

While removal of cancer causative agents from environment is was impossible, development of cancer chemoprevention was considerable importance. The concept of cancer chemoprevention referred to the prevention of cancer by using noncarcinogenic synthetic or naturally occurring products to inhibit or reverse the process of carcinogenesis (Sporn and Newton, 1979). A number of agents has been proved to have affects against chemical carcinogenesis in difference organs and could be classified into three categories according to the stage of carcinogenic process (Wattenberge, 1985) as follows :

(1) compounds preventing formation of ultimate carcinogens from their precursors.

(2) agents blocking at the initiation phase of cancer development.

(3) agents suppressing at the post-initiation phase of cancer development.

Many chemopreventive substances from medicinal plants have been identified (Boone *et al.*, 1990). Various kinds of Thai medicinal plants have been indicated to exert antimutagenicity and anticarcinogenecity including *Murdannia loriformis* (Hassk.) Rolla Rao et Kammathy. *M. loriformis* is the annual in family *Commelinacae.* It has 1.5 cm. width and 10 cm. length leaf. It's flower shows on the stem in blue or purple petal (Figure 1). *M. loriformis* comes from south Chinese and grow in sandy soil with the sunshine outline. This plant used in Chinese pharmacopeia for treatment and relive ailment of neoplasm. In Thailand, *M. loriformis* is used as a traditional drug among Thai people, to relief pain from bronchitis and cancer (Medicinal Plants in Siri Rukhachati Garden, 1992). Evidence has supported the induction DT-diaphorase activity in the murine hepatoma cell line, Hepa 1c1c7 (Vinitketkumneun *et al.*, 1996). Recently, the active glycosphingolipid has been isolated from *M. loriformis* extract and exerted cytotoxicity against human colon carcinomas and human breast cancer cell lines (Jiratchariyakul, 1995). However, the anticarcinogenic effect of *M. loriformis* extract in vivo has not been reported.

Bird (1987) reported the existence of aberrant crypts in murine colon, treated with a colon carcinogen., After that much attention has been drawn to them as a putative early lesion and a useful biomarker for studying colon carcinogenesis. This investigation addresses the study of inhibitory effects of *M. loriformis* extract on colon carcinogenesis. Present studies were carried out for the protective effect of examine the 80% ethanolic extract of *M. loriformis* against aberrant crypt foci and DNA adduct formation (O^6-methylguanine and N^7-methylguanine) in colonic mucosa or muscular layer of azoxymethane (AOM) exposed rats. The model of AOM-induced colon cancer in rodents, has been used as short-term assay for identifying chemopreventive agents for colon cancer (Pereira *et al.*, 1994; Pretlow *et al.*, 1991). The methylating agent, AOM, induced mutagenic and carcinogenic effects by forming two major of DNA adducts (Becker *et al.*, 1981). DNA adducts can be resulted directly from mutational events, and these potentially leading to cancer formation (Bos *et al.*, 1987).

Figure 1. *Murdannia loriformis* (Hassk.) Rolla Rao et Kammathy

1.2 Literature Reviews

1.2.1 Aberrant crypt foci (ACF) formation and colon carcinogenesis

Carcinogenesis is a complex, multistage process, presumably involving at the cellular molecular and morphological levels. The whole process can be divided into three main stages: initiation, promotion and progression. Each stage involves multiple events. Initially, there is widespread hyperplasia in the colonic epithelium followed by the development of tumors in the hyperplasia area. Progressive stages of small intermediate and late nonmalignant adenomas have been identified leading to malignant carcinomas and finally metastasis (Rumsby and Davies, 1994). The identification and quantification of early neoplastic lesions is very likely to be important in our understanding of cancer causation. Aberrant crypt foci (ACF) was first identified in the colon of carcinogen-treated rodents by a simple methodological approach (Bird, 1987).

In mammal, the crypts constitute a structural unit of the colonic epithelial. In the crypts, epithelial cells are produced constantly from a lower layer and migrated out of the crypt to the surface where they are extruded. The size, shape and cellularity depends heavily on the rate of proliferation, migration and loss of cells. Almost if not all, of the target cells of the colon specific carcinogens are the proliferative epithelial cells. Both direct and indirect acting carcinogens following metabolic activation may cause methylation to nuclear DNA of the colonic epithelial layer (Chang, 1984). Degenerative changes of the epithelial cells in the colonic crypts commenced within three hours after administration of a carcinogen and peaked at 24 hr. (Wargovich *et al.*, 1983). As a result of cell loss, there is a progressive decrease in the number of epithelial cells per crypts over 7 days compared to the pretreatment level (Chang, 1984). Among the remaining epithelial cells in the crypts, some recovered with error-free DNA, and some with error-prone DNA repairs. In the latter case, some are incompatible with survival and degenerated, while some are compatible with survival. During chronic treatment with carcinogens, occasional focal irregularities in of some crypts may occur in the proliferative compartment of the crypts.

1.2.2 Chemopreventive agents and colon cancer

Cancer prevention is the identification of preventable causes of cancer and the reduction of cancer incidence. Approaches to cancer prevention including primary, secondary and tertiary prevention. Primary prevention is the identification and elimination of cancer-causing environmental agents, such as chemicals, viruses and radiation exposure. Secondary prevention refers to the screening of individuals at increased risk for malignancy and the detection of neoplastic transformation at an early stage. Tertiary prevention refers to the utilization of specific pharmacologic agents or nutrients to prevent, delay or retard the development and progression of cancer (Krishnan and Brenner, 1996). Chemoprevention refers to the inhibition or reversal of carcinogenesis by the use of noncytotoxic agent drugs or nutrients to protect against the development and progression of mutant clones of malignant cells (Sporn, 1996). Currently, members of at least 25 different classes of chemicals have demonstrated chemopreventive activity (Wattenberg, 1992) i.e., vitamins and their analogues, trace metals, non-nutritive dietary components, hormones, drugs and many others. Several established carcinogen-induced rodent tumor models are being used to screen agents for chemopreventive activity (Boone *et al.*, 1992). These controlled experiments generally involve the administration of a single high dose of a classical carcinogen. Carcinogen-treated animal are given a chemopreventive agent at one or more stages along the carcinogenesis pathway, and the number of benign and malignant tumors are counted and histopathologically analyzed at the end of the experiment.

Colorectal carcinoma is an important, feasible and attractive target for chemoprevention because

(1) it is the forth common cancer in Thai males, the seventh common cancer in Thai female (Vatanasapt *et al.*, 1993) and the third most common cancer and cause of mortality in the United States and in other developed countries.

(2) there is a high mortality associated with the advanced disease.

(3) there is a well-described molecular carcinogenesis pathway

A variety of experimental models have been used to study the colon carcinogenesis pathway and its modulation. These include epidemiologic models (effect of bile acids and short-chain fatty acid), animal models of chemically induced colon cancer, histopathologic models (aberrant crypt foci assay), genetic models and transgenic and genetic knockout murine models (Krishnan and Brenner, 1996). The accumulated evidence derived from each of these models has increased the understanding of colon carcinogenesis. There is, however, no accepted method of classification of potential chemopreventive agents, because of the diversity of drug with chemopreventive properties. This is compounded by, poorly understood mechanisms and perhaps overlapping mechanisms of action of some of these drugs. Two provisional methods of classification are in use. In the first, the drugs are classified as agents that block or reverse initiation (class 1), agents that block or reverse promotion (class 2) and agents that block or reverse progression (class 3). The second classifies the drugs as blockers of mutagenic carcinogens or blockers of hyperproliferation. An ideal classification system has to be based on mechanisms of action of these drugs once they are well defined. A number of potential chemopreventives appear to have protective activity against colorectal carcinogenesis. These agents are listed in Table 1.

Table 1. Agents with colorectal chemopreventive activity (Krishnan and Brenner, 1996)

Class	Agents
Anti-inflammatory agents	Nonsteroidal anti-inflammatory drugs (aspirin, piroxicam, sulindac, sulindac sulfone), curcumin
Calcium	Calcium salts
Antioxidants	Retinoids, Carotenoids, Vitamin C and E
Polyamine inhibitors	Difluoromethyornithine
Dithiothiones	Oltipraz
Polyphenols	Ellagic acid
Miscellaneous	Vegetables, fruits, fiber, micronutrients /nonnutrients

Nonsteroidal anti-inflammatory drugs (NSAIDs): NSAIDs may suppress carcinogenesis by numerous potentially antagonistic pathways. This may be dependent on the specific NSAIDs used or the doses employed as well as the carcinogenic agents involved. Although the exact mechanism of the NSAIDs anticarcinogenic properties is not unknown, there are several potential chemopreventive pathways through which NSAIDs may exert their effects. NSAIDs may modulate apoptotic pathways in the gastrointestinal tract (Shiff *et al.*, 1996). NSAIDs reduced cell proliferation, increases the proportion of G_0/G_1 cells, and reduce the proporrotion in the S phase of in HT-29 colon adenocarcinoma cells (Piazza *et al.*, 1995).

Curcumin: Curcumin, a commonly used spice in Asia, is the major yellow pigment extracted from tumeric, the rhizome of the herb *Curcuma longa*. It is used as a cooking spice for many dishes. In India and Southeast Asia, curcumin has long been used as a treatment (as tumeric) for inflammation, skin wounds and tumors

(Ammon and Wahl, 1991). Curcumin functions as a scavenger of oxygen species (hydroxyl radical, superoxide anion, singlet oxygen) and interferes with lipid peroxidation (Kunchandy and Rao, 1990).

Calcium: Calcium suppresses AOM-induced ornithine decarboxylase (ODC) activity in rat colon (Arlow *et al.*, 1989). Because an increase of ODC activity has been observed in colon carcinomas, in colonic adenomas, and in tissue adjacent to adenomas, calcium-induced suppression suggests chemopreventive activity. Animals exposed to intrarectally instilled bile and fatty acids or a high-fat diet, showed an increase in colonic epithelial proliferation, which can be suppressed by calcium (Wargovich *et al.*, 1983). Epithelial cell proliferation has been suppressed by calcium in rodent colon perfused with bile acids, and animals fed with bile acid-rich diets or boluses of fat (Buset *et al.*, 1987). Proliferative activity of the colon epithelium has been shown to be reduced by oral administration of calcium carbonate. Another mechanism suggest that ionized calcium neutralized the effects of fatty acids and free bile acids and forms insoluble soaps that are not harmful to the colonic epithelium (Vogel and McPherson, 1989).

Polyamine inhibitors: In mammalian cells, polyamine synthetic inhibition by genetic mutation or pharmaceutical agents is associated with virtual cessation in cellular growth (Pegg, 1988). When exogenous polyamines are added, growth continues. These findings have led to several studies exploring the possibility that polyamine inhibitors may be useful as therapeutic and preventive agents in a variety of diseases involving deranged cell proliferation including cancer. The mechanism by which polyamines might prevent transformation is not known. It is possible that polyamines stabilize the DNA structure. ODC participates in carcinogenesis and is induced during cellular transformation by chemical carcinogens, viruses and oncogenes (Kelloff *et al.*, 1994). Blocking ODC with difluoromethyl ornithine (DFMO) or antisense RNA inhibited *v-src* transformation of rat 2 R fibroblasts. DFMO is a potent, irreversible inhibitor of ODC and it has *in vitro* antitumor activity. However, it has been found to be inactive when used as a systemic antineoplastic agent.

Dithiolthiones: Oltipraz is a synthetic dithiolthione. This compound has been shown to possess anticarcinogenic effects. The anticarcinogenic effects of cruciferous vegetables such as cauliflowers, brussel sprouts and cabbages may be due to the presence of dithiolthione. Oltipraz causes glutathione depletion in parasites but increases level of glutathione and other detoxification enzymes including glutathione-*S*-transferase in mammalian tissues (Kensler *et al.*, 1992). Oltipraz induces multi-phase II detoxification enzymes (GST, DT-diaphorase), which may contribute to its anticarcinogenic effects.

Polyphenols: Ellagic acid is a phenolic lactone present in several plants and fruits including strawberries, raspberries, grapes and nuts. The mechanisms of its chemopreventive effects may be due to its:

(1) function as a free radical scavenger by binding and inhibiting bay region diol epoxides of polycyclic aromatic hydrocarbons. Ellagic acid also prevents the binding of the carcinogen to DNA and enhances removal of the carcinogen (Wood *et al.*, 1982);

(2) prevention of methylation of DNA by carcinogens (Dixit and Gold, 1986);

(3) enhancement of antioxidant functions (glutathione peroxidase, catalase, quinone reductase) and phase II (GST) enzyme activities;

(4) inhibition of chemically induced lipid peroxidation;

(5) inhibition of TPA-induced epidermal ODC activity;

(6) inhibition of protein kinase C;

(7) increasing gap junction intercellular communication (Stoner and Mukhtar, 1995);

(8) induction of transcription of the antioxidant responsive element of the quinone reductase (QR) gene in transfection studies (Barch and Rundhangen, 1994).

Antioxidants: Free radicals are now known to play an important role in many diseases including aging (Slaga, 1989). The importance of free radicals in radiation carcinogenesis and free radicals and electrophiles in chemical carcinogenesis is also well recognized. Free radicals and reactive oxygen species are continuously produced *in vivo*. Consequently, organisms have evolved that possess not only antioxidant and electrophile defense systems to protect against them, but also repair systems that

prevent the accumulation of oxidatively-damaged molecules (Halliwell, 1993). Increased free radical levels and levels of electrophilic compounds have been associated with many disease conditions. Antioxidants, free radical scavengers and electrophilic scavengers may be very useful in cancer prevention, cardiovascular disease prevention, immune function augmentation, and increase the life span of man. Antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase are preventive antioxidants, because they eliminate species involved in the initiation of free radical chain reactions. Small molecule antioxidants, such as ascorbate, the tocopherols, glutathione and reduced coenzyme Q10 can repair oxidizing radicals directly, and therefore are chain-breaking antioxidants. It is well known that ascorbate and tocopherols function synergistically to protect membrane lipid from damage (Buettner, 1993).

Evidence accumulating and suggests that free radicals are important in all stages of chemical carcinogenesis. Several antioxidants have been found to inhibit all stages of carcinogenesis, whereas other are more effective against tumor initiation, promotion or progression. Many of antioxidants and related compounds such as phenolic antioxidants and vitamin C and E appear to be effective in counteracting the tumor initiating phase of carcinogenesis. This appears to be related to their antioxidant activity and their effect on carcinogen metabolism. In addition, many antioxidants such as the phenolic and polyphenolic antioxidants are potent inhibitors of the tumor promotion phase of carcinogenesis. Their effect on the free radical defense mechanisms, their antioxidizing activity and their effect on many critical events in tumor promotion, such as arachidonic acid metabolism, possibly explain why the antioxidants are potent inhibitors of tumor promotion. In some cases the antioxidants interact synergistically to inhibit carcinogenesis.

1.2.3 Drug metabolizing enzyme

Toxicology deals with exogenous compounds in the normal metabolism of the organism. Such compounds being referred to as foreign compounds or xenobiotics. However, many endogenous compounds, including metabolic intermediates such as glutamate or hormones are toxic when administered in unnaturally high doses. The

metabolism of xenobiotics, which is carried out by a wide range of relatively nonspecific enzymes, serve to increase the water solubility of foreign chemicals and their elimination from the body. This process generally consists of two phases (phase I and II). In phase I metabolism, a reactive polar group is introduced into the molecule, rendering it as a suitable substrate for phase II reactions. Phase I reactions include the well-known cytochrome P450-dependent monooxygenations as well as reductions, hydrolysis, etc. Phase II reactions include all of the conjugation reactions in which a polar group on the toxicant is combined with an endogenous compound such as glucuronic acid, glutathione, sulfate, etc., to form a highly water-soluble conjugate that can be readily eliminated from the body.

Foreigned compounds can be substrates, inhibitors or inducers of the enzymes that metabolize them and, and not in frequently, serve in more than one of these roles. Since the enzymes in question are nonspecific, numerous interaction between foreign compounds are possible. These may be synergistic or antagonistic and may have a profound effect on the expression of toxicity. Depending upon the compounds and the enzymes involved in the particular interaction, the effect can be an increase or a decrease in either acute or chronic toxicity. The overall metabolic scheme for potentially toxic xenobiotics is outlined in Figure 2.

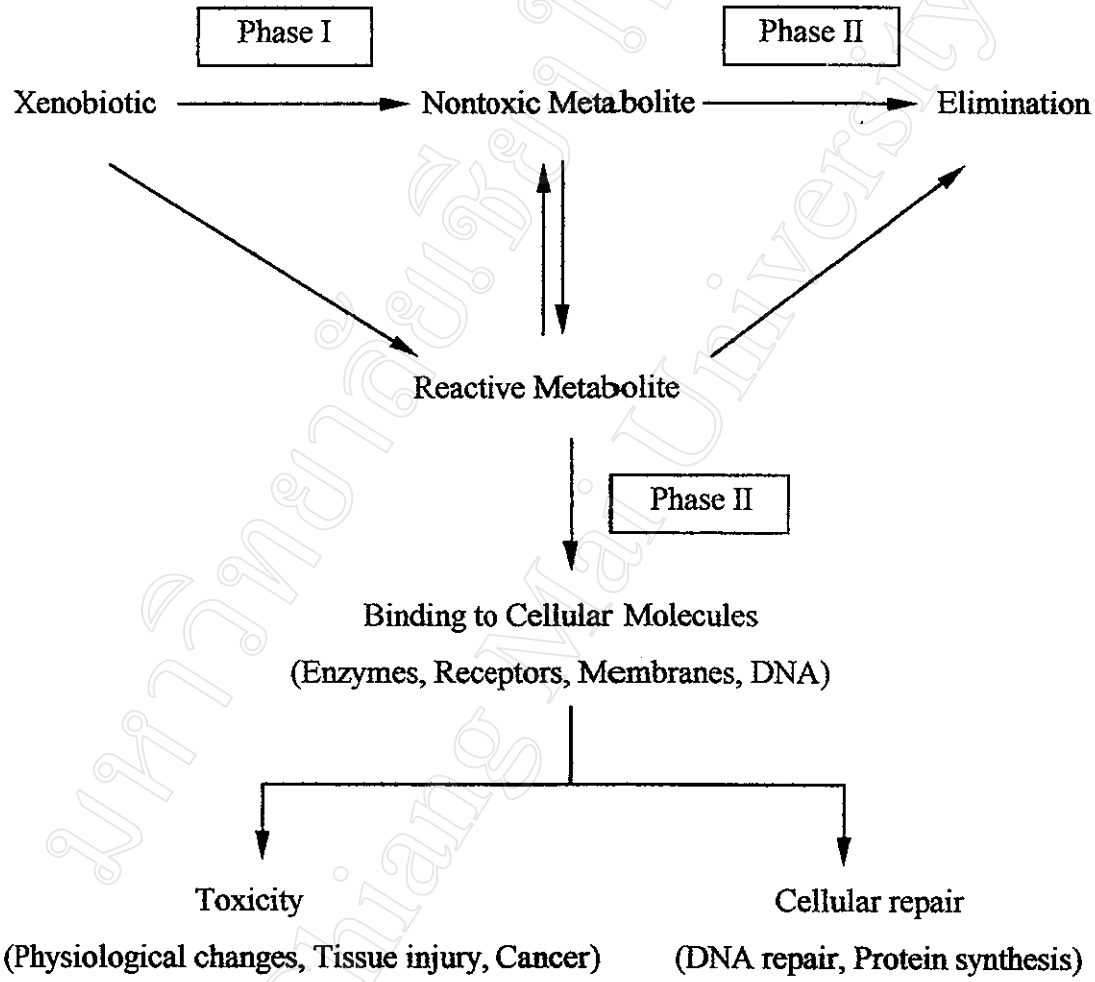

Figure 2. The relationship between metabolism, activation, detoxification and toxicity of a chemical (Levi, 1994).

1.2.3.1 Phase I enzymes

Cytochrome P450: While most, if not all, of the enzymes involved in xenobiotic metabolism can form reactive metabolites (Table 2), the enzyme systems most frequently involved in the activation of xenobiotics catalyze oxidation reactions. The cytochrome P450 monooxygenases (P450) are for the most important enzymes involved in oxidation of xenobiotics. This is a result of the abundance of P450, especially in the liver, the numerous isozymes of P450, each with a broad substrate specificity, and the ability of P450 to be induced by xenobiotics which are often its substrates. In addition to metabolism by the liver, P450 is known to be involved in xenobiotic metabolism and activation reactions in extrahepatic organs such as the lung, kidney, and skin, sometimes resulting in toxicity. This enzyme system consists of a number of families, each with one or more distinct proteins which have different as well as overlapping substrate specificities. Sometimes a chemical may be preferentially metabolized by only one P450; frequently, however, a chemical serves as substrate for more than one P450. These differences in metabolism may have profound effects on the toxicity of the chemical.

1.2.3.2 Phase II enzymes

Conjugation by the phase II enzymes such as glutathione S-transferases (GST), glucuronyl transferases and sulfotransferases is generally considered to be a detoxication process.

Sulfate conjugation: Sulfation is now recognized as one of the major Phase II conjugation reactions in the biotransformation of a range of drugs and xenobiotic compounds as well as many endogenous compounds. Recent studies have greatly increased the range of compounds recognized as substrates for conjugation. One reason for the large number of different compounds that are sulfated, is that many drugs and xenobiotics are first metabolized by phase I or oxidative enzyme systems to generate phenols and alcohols, which may be conjugated with sulfate. Many compounds already posses the appropriate chemical structures for direct conjugation with sulfate.

The major physiologic result of conjugation of small xenobiotic compounds with the charged sulfonate moiety, is a decrease in their biologic activity and an increase in their hydrophilicity. The increased water solubility of the sulfate conjugate almost always leads to an increase in their rate of excretion into the urine or bile. These properties represent the major function of sulfation in xenobiotic metabolism.

Glucuronidation represents a major drug metabolizing reaction catalyzed by a superfamily of UDP-glucuronosyl transferase (UGT). UGT isozymes are located in the endoplasmic reticulum and nuclear membrane of hepatocytes and in various extrahepatic tissues such as intestine, kidney and olfactory epithelium. In general, they convert hydrophobic endogenous or xenobiotics into less pharmacologically active, water-soluble products that can be excreted. Endogenous substrates include bilirubin, steroid hormones and fat soluble vitamins. Xenobiotic substrates include many drugs, plant constituents, environmental pollutants and carcinogens.

Table 2. Enzymes important in catalyzing metabolic activations.

Type of Reaction	Enzyme
Oxidation	Cytochrome P450
	Prostaglandin synthetase (PGS)
	Flavin-containing monooxygenase
	Alcohol and aldehyde dehydrogenase
Conjugation	Glutathione transferase
	Sulfotransferase
	Glucuronidation
De-conjugation	Cysteine S-conjugate β-lyase
Gut microflora	Hydrolases
	Reductases

Glutathione conjugation: The glutathione *S*-transferases are multifunctional proteins that serve several cellular functions. A prominent function, which is associated with their widely recognized cytoprotective role, is the catalysis of the reaction of glutathione with electrophiles at the first enzymatic step in mercapturic acid biosynthesis. The liver is the major site of glutathione *S*-conjugate formation with nephrotoxic haloalkenes. The glutathione *S*-conjugates are excreted in the bile and pass into the small intestine. In the bile duct and in the intestine, some of the glutathione *S*-conjugates are hydrolyzed to the corresponding L-cysteinylglycine and L-cysteine *S*-conjugates.

1.2.4 AOM metabolism

Azoxymethane (AOM), principally a colon carcinogen in rodents (Lijinsky *et al.*, 1985) is metabolically derived from 1,2-dimethylhydrazine via azomethane as an intermediate (Fiala, 1977). AOM is methylated at hydroxyl group to be methylazoxymethanol (MAM) by cytochrome P450 IIE1 in the liver (Sohn *et al.*, 1991). MAM, the proximate metabolite of AOM, has a half life of 12 hr. under physiological conditions (Feinberg and Zedeck, 1980). Because of its relatively higher stability, MAM can be readily transported to extrahepatic organs such as the colon via the blood stream for further activation. Alternatively, MAM may conjugate with glucuronic acid immediately upon formation and be transport via bile to the colon (Weisburger, 1971). It would then be hydrolyzed by bacterial β-glucuronidase to generate free MAM (Mutsumoto *et al.*, 1979). The free MAM will cooxidize with prostaglandin synthesis. For example the lipoxygenesis system (Craven *et al.*, 1985) yields a reactive compound, methylcarbonium ion that can methylate DNA molecule to O^6-methylguanine or N^7-methylguanine adducts (Figure 3).

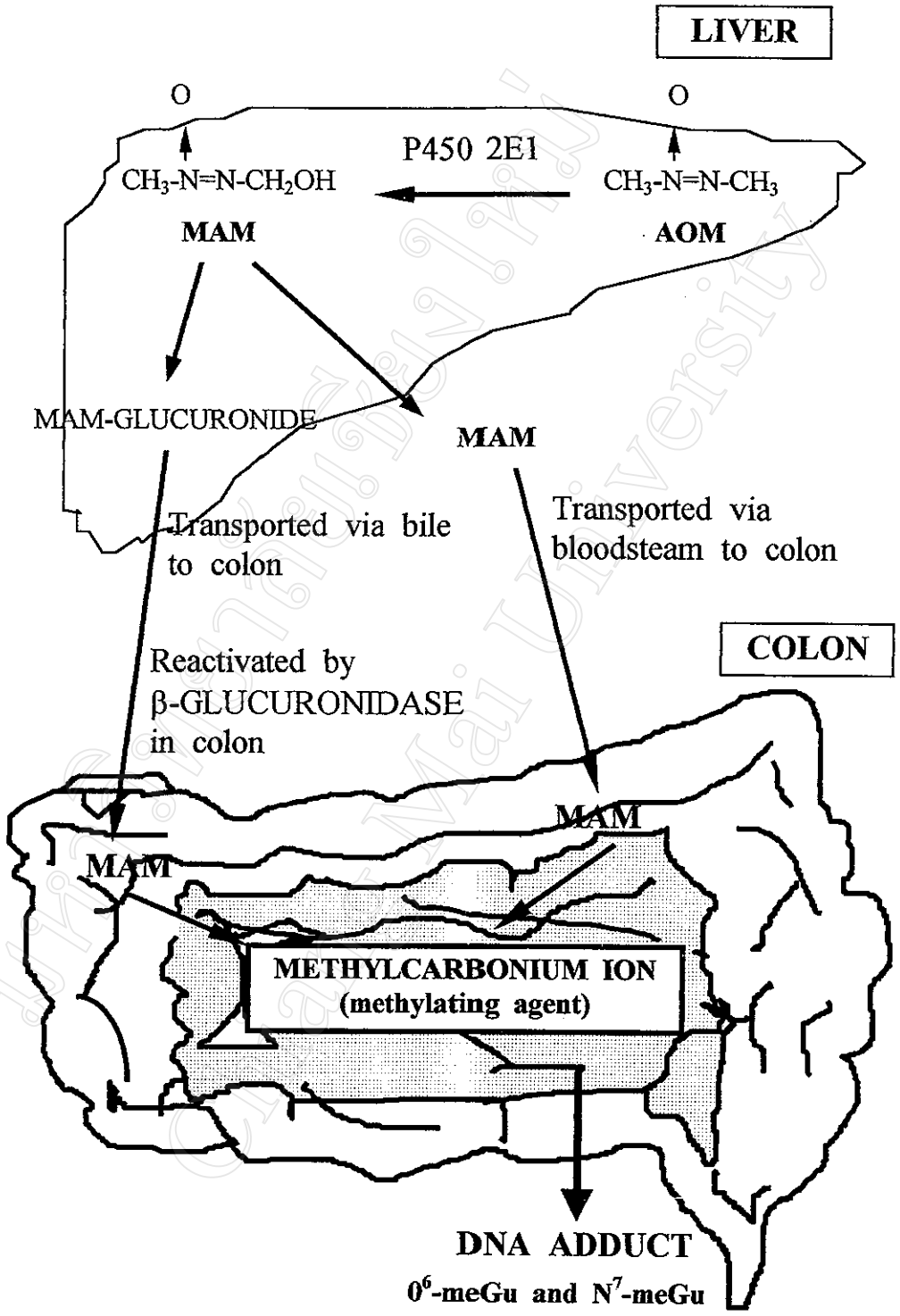

Figure 3. Azoxymethane metabolic pathway (Sohn *et al.*, 1991)

1.3 Proposes of the study

1. To investigate effects of *M. loriformis* 80% ethanol extract on AOM-induced ACF formation at initiation stage and promotion stage of colon carcinogenesis

2. To investigate effects of *M. loriformis* extract on AOM-induced DNA adduct (N^7-methylguanine and O^6-methylguanine) formation

3. To study antioxidant activity of *M. loriformis* extract

4. To study effects of *M. loriformis* extract on some drug-metabolizing enzymes activity