# CHAPTER 4
# DISCUSSION AND CONCLUSION

It is well documented that Stevia and its products, Stevioside are used as a sugar substitute in some parts of the world (Fujita and Edahiro, 1979; Soejarto, *et al*, 1983). stevioside, the sweet compounds in Stevia, is 300 times sweeter than sucrose and is presently employed as a non-caloric sweetener. Although, many of the research data indicated that Stevia and Stevia products were innocuous. However, the aglycone of stevioside, steviol was found to be highly mutagenic toward *Salmonella typhimurium* TM677 (Pezzuto, *et al*, 1985a) and was highly toxic to both dams and fetuses in hamsters (Wasuntarawat, *et al*, 1998). Thus, the sweetening properties and the potential pharmaceutical activities of Stevia sweetener have received worldwide attention and also under consideration in Thailand. Additives in food are incorporated into the human body throughout life. Many kinds of cancer have a long latency. Therefore, the duration of chronic toxicity and carcinogenicity tests of food additives in rats is necessary to confirm for security. The present study has investigated the effect of Matoom (*Aegle marmelos Corr.*) juice containing Stevia extract as sweetener on aberrant crypt foci (ACF) formation in colon, activity of AST and ALT in serum, GSH content, GST activity and subunit composition in the liver and intestinal mucosa in male Wistar rats after 35 or 100 days administration of Matoom juice containing Stevia extract as sweetener.

Stevia extracts in the form of steviosides are hundreds of times sweeter than sugar, so only tiny amounts are used. If excessive amounts are used can result in excessive sweetness and has a varying degrees of bitterness associated with the sweet. The optimum concentrations for use in food and beverage are in the range of 0.005 – 0.06% by weight (Daniel B, 1992). In this study, various concentrations of

stevioside from 0.006 – 0.3%w/v was mixed in Matoom juice. These concentrations have a good sweet taste without bitterness for human.

Progress in the field of diet and colon cancer would be greatly enhanced by the development of a methodology which allowed for the identification and quantification of the early precursor lesions of colon cancer. It was hypothesized that aberrant crypts foci formation represented preneoplastic lesion that have been purposed to be a biomarker for a short-term screening assay for colon carcinogenesis (Pereira, *et al*, 1994). Irrespective of the type of carcinogen or species of animal studied, it is generally believed that colon tumorigenesis is a focal and sequential process originating in a single crypt and involving a clonal proliferation of transformed cells (Chang, 1984). Thus, the induction of ACF and sequential analysis of their growth pattern with respect to size and histological features will be valuable in the identification of potential colon tumor initiators and promoters (Bird, *et al*, 1989). The effects of Matoom juice containing Stevia extract as sweetener on the number of ACF in the initiation and promotion stage were evaluated in this study. It was found that treated rats with Matoom juice containing various concentrations of Stevia extract as sweetener from 0.2-10%w/v and Stevia extract 10.0%w/v in distilled water were not able to induce ACF formation in colon both at the initiation and promotion stage (Table 1 and 2). At initiation stage, ACF formation was observed in only rats treated with AOM (positive control group). Table 1 has shown that the mean total number of ACF in group 2 was 142.50 aberrant crypts and no ACF formation was presented in rats treated with Matoom juice containing various concentrations of Stevia extract and Stevia extract at 10.0% in distilled water. At promotion stage, Stevia extract from 0.2-10%w/v and Stevia extract at 10.0% w/v in distilled water were not able to promote ACF formation in rats colon. Not only ACF formation was observed in rats treated with AOM but also single tubular adenoma at proximal segment 2 in 8 rats in this group. The mean total number of ACF in rats treated with AOM was 407.75 aberrant crypts (Table 2). The result of ACF formation of this study indicated that there was no toxic caused by dietary administration of Matoom juice which contained Stevia extract as

sweetener. A continuous treatment with the Matoom juice containing various concentrations of Stevia extract from 0.2-10%w/v and Stevia extract at 10.0%w/v in distilled water as long as 100 days did not have potential in carcinogenicity of colon cancer. These finding was related with the observations of Toyoda, *et al* who reported that subchronic toxicity study of stevioside showed no significantly alteration in the development of neoplastic or non-neoplastic lesions in any organ or tissue (Toyoda, *et al*,1997).

Elevated levels of serum enzymes are the indication of cellular leakage and the loss of functional integrity of cell membrane (Drotman and Lawhorn, 1978). Damage of liver cells cause leakage of cellular enzymes into serum. Significant rise in serum transaminase concentration (AST and ALT) could be taken as an index of liver damage. The amount of AST and ALT are directly related to the number of cells affected by the disease or injury (Jacobs and David, 1996). Although not specific for liver disease, it can be used in combination with other enzymes to monitor the course of various liver disorders. Determination of AST and ALT also assists in early recognition of toxic hepatitis that results from exposure to drugs toxic to the liver, like acetaminophen and cholesterol (Pagana, *et al*, 1998). It is also reported that the chronic thioacetamide exposure produced cirrhosis in rats and caused significant increase in serum AST and ALT (Chieli and Nalvadi, 1985). The present study has been focused on alteration in serum AST and ALT to possible beneficial effect of the use of Stevia extract mixed in Matoom juice effect on liver diseases. The results indicated that there was no modification effect on serum AST and ALT by drinking Matoom juice which used Stevia extract as sweetener (Table 3 and 4). The serum AST and ALT activities were increased in serum of rats treated with AOM when compared with non-treated group. In 100 days treatments, different ALT activities of positive control were slightly increased in serum but no significant from normal control. According to the studied of Kenneth (Kenneth, 2000), AST was more sensitive than ALT and ALT activity in hepatic injury will return to baseline level in 3-4 weeks. The result from this study showed that daily exposure of Matoom juice containing Stevia

extract as sweetener had non-toxic effect to the rat liver. Similar results were reported by Simoliar, *et al*, that chronic effects of sweetening agent from Stevia (10 months) did not produce a significant effect on the parameters of metabolic processes and morphological picture of the internal organs involved hepatic tissues in tested rats (Simoliar, *et al*, 1992).

Natural and synthetic agents play an important role in the risk of cancer development and in the prevention of cancer (McLellan and Bird, 1991). Many types of chemical agents may alter the xenobiotic-metabolizing enzymes in carcinogen metabolism. Chemical carcinogens may contain common biological property; such as they are inducers of enzymes involved in the metabolism of carcinogens and other xenobiotics in animal tissues and cell culture (Prochaska and Talalay, 1988).

Glutathione is an important endogenous antioxidant system that is found in particularly high concentration in liver and it is known to have key functions in protective processes. The reduced form of GSH becomes readily oxidized to GSSG on interacting with free radicals. Excessive production of free radicals resulted in the oxidative stress, which leads to damage of macromolecules e.g. lipids, and can induce lipid peroxidation *in vivo* (Sinclair, *et al*, 1991). The antioxidant properties of GSH and its ability to conjugate with reactive electrophilic metabolites and xenobiotics suggest that this molecule might be significant in protecting the cell against various toxic insults (Jafoby, 1977; Bast and Haenen, 1988). Intracellular GSH status appears to be a sensitive indicator of the cell's overall health, and of its ability to resist toxic challenge. Oxidative stressors such as cigarette smoke, atmospheric pollutants, and other inhaled environmental toxins results in depletion of GSH and other antioxidants from the lungs. (Pacht, *et al*, 1991; Bunnell and Pacht, 1993). Moreover, thioacetamide, a potent hepatotoxin and carcinogen in rats (Fitzhugh and Nelson, 1948) produced the depletion in GSH concentration. In this study, GSH contents in liver and small intestinal mucosa of rats treated with AOM were decreased more than the non-treated groups (Table 5 and 6). Administration of either 35 or 100 days of Matoom juice containing Stevia extract as sweetener could not have any effect on GSH contents in all organs

examined. GSH conjugation is considered to be a protective mechanism against the harmful effects of many xenobiotic substances, the degree of protection, or conversely susceptibility, depending on the level of GSH and the present of the appropriate GST isoenzymes. Alterations in the GST activities, isoenzymes levels and GSH-dependent metabolism were also used to monitor the susceptibility of organs or tissues to the toxicity of various xenobiotics and toxicants in this study.

Glutathione-S-transferase (GSTs) are enzymes that catalyze the conjugation of electrophilic compounds with GSH, resulting in soluble complexes that are generally more hydrophilic and less cytotoxic. In this study, enhancement of GST activity in hepatic and small intestinal mucosa was observed in rats treated with AOM (Table 7 and 8). These results likely suggest that administration of Matoom juice containing Stevia extract as sweetener may not possibly change the activity of the phase II xenobiotic metabolizing enzymes, such as GST. In contrast to Pezzuto, *et al* who reported that steviol, the aglycone metabolites of stevioside, uncovered as a potentially mutagenic chemical and induces GST activity in mice (Pezzuto, *et al*, 1986). Wingard, *et al* sugested that stevioside is swiftly transformed into steviol by the action of anaerobic microorganism in the intestine (Wingard, *et al*, 1980). However, Pezzuto, *et al*, who carried out a comprehensive study in which stevioside was incubated for up to 3 months with various intestinal micro-organisms; no steviol production could be detected. Studies with steviol-17[$^{14}$C] showed that 96% of an oral dose was eliminated in the faeces of rats, and that the aglycone is completely absorbed through the lower bowl (Wingard, *et al*, 1980). The bacterial flora of mammalians is the most intimate portion of their biological environment and mediates many interactions with the chemical environment. The genera of gastro-intestinal microflora in human related to rodent bacteria but different in strain and major population. Mcdonald showed that the most common organisms in gastro-intestinal of human were enterobacteria, enterococci, lactobacilli, clostridia, bacteroides and bifidobacterium. In contrast with main groups of gastrointestinal bacteria in rodents, that composed of four major

groups of bacteria; compylobacteriosis, selemonas, borrelia and spiral bacteria (Mcdonald, 1989). As yet, stevioside metabolism has not been investigated in humans.

Enhancing activity of GST detoxification potential could increase the capacity to withstand the burden of daily exposure to toxicants and carcinogen (Carr, 1985). The concentration of reactive metabolites of environmental pollutants, plant toxins and drugs will be decreased more rapidly when GST levels were high (Hatono, *et al*, 1996). In addition, the levels of GST, a family of detoxification enzymes consisting of class alpha, mu, pi and theta isoforms, were inversely correlated with cancer risk (Esther, *et al*, 1998). They are expressed in a tissue and development-specific fashion. It is expected that individuality in the levels of expression of specific isoenzymes may lead to individuality in the response to external toxins and carcinogens. For instance, the well-known carcinogens aflatoxin B1 and benzo[a]pyrene are detoxified predominantly by class-alpha and class-mu GSTs, respectively (Coles and Ketterer, 1990). GST Ya subunit gene expression is induced in mammalian tissues by two types of chemical agents: (i) planar aromatic compounds (e.g., 3-methylcholanthrene, beta-naphthoflavone, and 2,3,7,8-tetrachlorodibenzo-p- dioxin) and (ii) electrophiles (e.g., trans-4-phenyl-3-buten-2-one and dimethyl fumarate) or compounds easily oxidized to electrophiles (e.g., tert-butylhydroquinone) (Friling, *et al*,1990). In the present study, the increase of GST alpha class (both Ya and Yc subunits) and decrease of mu class were observed in rats received AOM when compared with non-treated rats. The levels of these two classes by administration of Matoom juice containing Stevia extract as sweetener were not different from non-treated rats. GST theta class levels in livers of rats treated with AOM were decreased but slightly increased in rats treated with Matoom juice containing Stevia extract as sweetener. This result indicated that Matoom juice containing Stevia extract as sweetener could not alter the levels of GST isoenzymes. Alterations in the expression of specific forms of GSTs in clinical disease and in experimental models were used as biomarkers of exposure and as modulator to therapeutic benefit. Zhong, *et al*. Reported that the risk of colorectal cancer was associated with the *GSTM1* and *GSTT1* null genotype expression (Zhong, *et al*, 1993).

In mouse, GST-mu was detected in all tissues investigated but was markedly decreased in mouse colon adenocarcinoma Co38, compared to normal colon. It is possible that GST-mu could play a key role in the malignant phenotype of this mouse colon (Massaad, *et al*, 1992). In addition, expression of GST isoenzymes especially pi and alpha isoenzymes have often been implicated in the development of resistance towards various toxic insults (Wareing, *et al*, 1993). Raza *et al*, reported that expression of GST isoenzymes especially GST alpha and pi was slightly higher in the brain of treated animals and suggested a mechanism for protection against the toxic effects of gasoline or its metabolic products in this tissues (Raza, *et al*, 1995). A decrease in hepatic GSH concentration and an increase in GST activity were observed in diabetic rats (Haider, *et al*, 1996). An increase in GST catalytic activity in diabetic rats was in agreement with an observation of apparent increased expression of GST alpha and pi by Western blot analysis. It has been shown that the multiple forms of the GST have many roles in protecting the cell from a wide range of toxic insults, which is benificial to the normal tissue.

In conclusion, the present study demonstrated that administration of Matoom juice containing Stevia extract as sweetener as long as 100 days could not able to induce preneoplastic lesions in colons and did not have any carcinogenic or other adverse effects in xenobiotic metabolism enzymes in male Wistar rats. These results, and those of other studies of Stevia extract, indicated that it may be safe to use as natural sweetener with low calorific value. It is non-toxic and also non-carcinogenic.